

APPLICATION FOR LETTERS PATENT

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RAPID IMMUNOSELECTION CLONING METHOD

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RAPID IMMUNOSELECTION CLONING METHOD

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This application is a divisional of co-pending U.S. Patent Application No. 07/983,647, filed December 1, 1992, which is a continuation-in-part of co-pending U.S. Patent Application Serial Number 553,759, filed July 13, 1990; which is a continuation-in-part of USSN 07/498,809 filed March 23, 1990; which is a continuation-in-part of USSN 379,076, filed July 13, 1989; which is a continuation-in-part of co-pending U.S. Serial Number 160,416, filed February 25, 1988. Each of these predecessor applications and all references cited herein are incorporated by reference in their entirety.

Background

15 A basic tool in the field of recombinant genetics is the conversion of poly(A)⁺ mRNA to double-stranded (ds) cDNA, which then can be inserted into a cloning vector and expressed in an appropriate host cell. Molecular cloning methods for ds cDNA have been reviewed, for example, by Williams, "The Preparation and Screening of a cDNA Clone Bank," in Williamson, ed., Genetic Engineering, Vol. 1, p. 2, Academic Press, New York (1981);

20 Maniatis, "Recombinant DNA", in Prescott, ed., Cell Biology, Academic Press, New York (1980); and Efstratiadis et al., "Cloning of Double-Stranded DNA," in Stelo et al., Genetic Engineering, Vol. 1, p. 15, Plenum Press, New York (1979).

25 A substantial number of variables affect the successful cloning of a particular gene and cDNA cloning strategy thus must be chosen with care. A method common to many cDNA cloning

strategies involves the construction of a "cDNA library" which is a collection of cDNA clones derived from the total poly(A)⁺ mRNA derived from a cell of the organism of interest.

5 A mammalian cell may contain up to 30,000 different mRNA sequences, and the number of clones required to obtain low-abundance mRNAs, for example, may be much greater. Methods of constructing genomic eukaryotic DNA libraries in different expression vectors, including bacteriophage lambda, cosmids, and viral vectors, are known. Some commonly used methods are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, New York (1982).

10 Once a genomic cDNA library has been constructed, it is necessary to isolate from the thousands of host cells the cell containing the particular human gene of interest. Many different methods of isolating target genes from cDNA libraries have been utilized, with varying success. These include, for example, the use of nucleic acid probes, which are labeled mRNA fragments having nucleic acid sequences complementary to the DNA sequence of the target gene. When this method is applied to cDNA clones of abundant mRNAs in transformed bacterial hosts, colonies hybridizing strongly to the probe are likely to contain the target DNA sequences. The identity of the clone then may be proven, for example, by in situ hybridization/selection (Goldberg et al., Methods Enzymol., 68:206 (1979)) hybrid-arrested translation (Paterson et al., Proceedings of the National Academy of Sciences, 74:4370 (1977)), or direct DNA sequencing (Maxam and Gilbert, Proceedings of the National Academy of Sciences, 74:560 (1977); Maat and Smith, Nucleic Acids Res., 5:4537 (1978)).

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30 Such methods, however, have major drawbacks when the object is to clone mRNAs of relatively low abundance from cDNA libraries. For example, using direct in situ colony hybridization, it is very difficult to detect clones containing cDNA complementary to mRNA species present in the initial library

population at less than one part in 200. As a result, various methods for enriching mRNA in the total population (e.g. size fractionation, use of synthetic oligodeoxynucleotides, differential hybridization, or immunopurification) have been developed and are often used when low abundance mRNAs are cloned. Such methods are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, supra.

Many functional eukaryotic proteins initially exist in the form of precursor molecules which contain leader or signal sequences at their N-terminal ends. These leader sequences bind to the cell membrane and draw the remainder of the protein through the lipid bilayer, after which the signal sequence is cleaved from the protein by a signal peptidase enzyme. The protein thus functions only after secretion from the cells (for example, insulin, serum albumin, antibodies, and digestive tract enzymes), or after the proteins have been anchored to the outer surface of a cell membrane (for example, histocompatibility antigens).

The cell surface antigens characteristic of mammalian T lymphocytes are additional examples of proteins that anchor to the cell surface. In mammals, certain cells derived from bone marrow mature into lymphocytes, which are present in the lymphoid organs, including the thymus, spleen, lymph nodes, and lymphoid aggregates, and also circulate actively through the blood and lymph systems. Mature lymphocyte cells may be divided into two populations: thymus-dependent (T) lymphocytes and thymus-independent (B) lymphocytes. T lymphocytes migrate to the interior of the thymus, where they undergo differentiative proliferation. During their differentiation process, they express characteristic cell surface membrane alloantigens, including Thy-1, TLA, gv-1, Ly-1, Ly-2, Ly-3, and Ly-5. As they mature, T lymphocytes lose the TLA antigens and some of the Thy-1 antigens, and gain histocompatibility antigens, acquiring the membrane conformation typical of the recirculating T lymphocytes. This is described, for example, by Mota, "Activity of Immune

Cells," in Bier et al., eds., Fundamentals of Immunology, 2d Ed., Springer-Verlag, Berlin, pp. 35-62 (1986).

5 T lymphocytes are involved indirectly in the formation of antibodies and their activities thus have required complex analysis of cell function, rather than simple antibody titer measurement. Partly due to this, their importance in development of immunologic competence was not recognized until relatively recently. Mature T lymphocytes synthesize and express an unique pattern of surface glycoprotein antigens which serve as markers for identification of different T lymphocyte subpopulations, including T helper cells, T suppressor cells, and T cytotoxic cells. Each of these subpopulations plays a very important role in regulating the immune system. (Mota, supra).

10 In humans, the functional and phenotypic heterogeneity of T lymphocytes is well accepted. Two major subpopulations are known: effector T cells mediating cellular immunity; and regulator T cells containing helper and suppressor T lymphocytes. These two subpopulations have been defined with heteroantisera, autoantibodies, and monoclonal antibodies directed at cell surface antigens. For example, earlier in their development, human lymphoid cells in the thymus express an antigen designated T11 which reacts strongly to a monoclonal antibody designated Cluster of Differentiation 2 (CD2), and react slightly with monoclonal antibody CD5 to cell surface antigen T1. During maturation, these cells lose T11 (CD2) and acquire three new antigens defined by monoclonal antibodies CD4, CD8, and CD1. With further maturation, the thymocytes cease to express cell surface antigens reactive with monoclonal antibody CD1, express the T3 antigen reactive with monoclonal antibody CD3, and then segregate into two subpopulations which express either T4 (CD4), or T8 (CD8) antigen. Immunologic competence is acquired at this stage, but is not completely developed until thymic lymphocytes migrate outside the thymus. (Mota, supra.) In contrast with the majority of thymocytes, circulating T lymphocytes express the T1 (CD5) and T3 (CD3) antigens. The T4 (CD4) antigen is present on

approximately 55-65% of peripheral T lymphocytes, whereas the T8 (CD8) antigen is expressed on 20-30%. These two subpopulations correspond to helper and to suppressor and cytotoxic T cells, respectively.

5 In addition to providing a convenient means of distinguishing T lymphocyte subpopulations, these cell surface antigens are important for mature T cell activation and effector function. T cell activation involves a complex series of cell surface interactions between the T cell and the target cell or stimulator cell in addition to binding of the T cell receptor to its specific antigen.

For example, CD2, the human T cell erythrocyte receptor, allows thymocytes and T-lymphocytes to adhere to target cells (e.g., erythrocytes) and to thymic epithelium. This occurs via a specific molecular ligand for CD2, designated LFA-3, in humans, which is a widely distributed surface antigen. This phenomenon has long been employed to detect, assay and purify human cells producing antibodies to sheep erythrocytes and serves as the basis for the E-rosette test, first described by Zaalberg, Nature 202:1231 (1964). CD2/LFA-3 interactions also have been shown to mediate cytolytic target conjugation (Shaw et al., Nature 323:262-264 (1986), and the mixed lymphocyte reaction (Martin et al., J. Immunol. 131:180-185 (1983). Anti-CD2 monoclonal antibodies can directly activate peripheral T-lymphocytes via an antigen-independent pathway (Meuer et al., Cell 36:897-906 (1984)), indicating an even wider immunoregulatory role for CD2.

Recognition that T lymphocytes are the main effectors of cell-mediated immunity and also are involved as helper or suppressor cells in modulating the immune response has resulted in a significant contribution to the increasing practical application of clinical immunology to medicine. The scope of this application includes defense against infections, prevention of diseases by immunization, organ transplantation, blood banking, and treatment of deficiencies of the immune system and

a variety of disorders that are mediated by immunologic mechanisms. Moreover, immunologic techniques frequently are used in the clinical laboratory, as in the measurement of hormones and drugs. Clinical immunology is described, for example, in Weir, ed., Handbook of Experimental Immunology in Four Volumes: Volume 4: Applications of Immunological Methods in Biomedical Sciences, 4th Ed., Blackwell Scientific Publications, Oxford (1986); Boguslaski et al., eds., Clinical Immunochemistry: Principles of Methods and Applications, Little, Brown & Co., Boston (1984); Holborow et al., eds., Immunology in Medicine: A Comprehensive Guide to Clinical Immunology, 2d Ed., Grune & Stratton, London (1983); and Petersdorf et al., eds., Harrison's Principles of Internal Medicine, 10th ed., McGraw-Hill, New York, publisher, pp. 344-391 (1983). Clearly, a more thorough understanding of the proteins which mediate the immune system would be of significant value in clinical immunology.

Use of mammalian expression libraries to isolate cDNAs encoding mammalian proteins such as those described above would offer several advantages. For example, the protein expressed in a mammalian host cell should be functional and should undergo any normal posttranslational modification. A protein ordinarily transported through the intracellular membrane system to the cell surface should undergo the complete transport process. A mammalian expression system also would allow the study of intracellular transport mechanisms and of the mechanism that insert and anchor cell surface proteins to membranes.

One common mammalian host cell, called a "COS" cell, is formed by infecting monkey kidney cells with a mutant viral vector, designated simian virus strain 40 (SV40), which has functional early and late genes, but lacks a functional origin of replication. In COS cells, any foreign DNA cloned on a vector containing the SV40 origin of replication will replicate because SV40 T antigen is present in COS cells. The foreign DNA will replicate transiently, independently of the cellular DNA.

With the exception of some recent lymphokine cDNAs isolated
 by expression in COS cells (Wong, G.G., et al., Science 228:810-
 815 (1985); Lee, F. et al., Proceedings of the National Academy
 of Sciences, USA 83:2061-2065 (1986); Yokota, T., et al.,
 5 Proceedings of the National Academy of Sciences, USA 83:5894-5898
 (1986); Yang, Y., et al., Cell 47:3-10 (1986)), however, few
 cDNAs in general are isolated from mammalian expression
 libraries. There appear to be two principal reasons for this:
 First, the existing technology (Okayama, H. et al., Mol. Cell.
 10 Biol. 2:161-170 (1982)) for construction of large plasmid
 libraries is difficult to master, and library size rarely
 approaches that accessible by phage cloning techniques. (Huynh,
 T. et al., In: DNA Cloning Vol. I, A Practical Approach, Glover,
 D.M. (ed.), IRL Press, Oxford (1985), pp. 49-78). Second, the
 existing vectors are, with one exception (Wong, G.G., et al.,
Science 228:810-815 (1985)), poorly adapted for high level
 expression, particularly in COS cells. The reported successes
 with lymphokine cDNAs do not imply a general fitness of the
 methods used, since these cDNAs are particularly easy to isolate
 from expression libraries. Lymphokine bioassays are very
 sensitive ((Wong, G.G., et al., Science 228:810-815 (1985); Lee,
 F. et al., Proceedings of the National Academy of Sciences, USA
 83:2061-2065 (1986); Yokota, T. et al., Proceedings of the
National Academy of Sciences, USA 83:5894-5898 (1986); Yang, Y.
 et al., Cell 47:3-10 (1986)) and the mRNAs are typically both
 abundant and short (Wong, G.G. et al., Science 228:810-815
 (1985); Lee, F., et al., Proceedings of the National Academy of
Sciences, USA 83:2061-2065 (1986); Yokota, T., et al.,
 20 Proceedings of the National Academy of Sciences, USA 83:5894-5898
 (1986); Yang, Y., et al., Cell 47:3-10 (1986)).
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Thus, expression in mammalian hosts previously has been most
 frequently employed solely as a means of verifying the identity
 of the protein encoded by a gene isolated by more traditional
 cloning methods. For example, Stuve et al., J. Virol. 61(2):327-
 35 335 (1987), cloned the gene for glycoprotein gB2 of herpes
 simplex type II strain 333 by plaque hybridization of M13-based

recombinant phage vectors used to transform competent E. coli JM101. The identity of the protein encoded by the clone thus isolated was verified by transfection of mammalian COS and Chinese hamster ovary (CHO) cells. Expression was demonstrated by immunofluorescence and radioimmunoprecipitation.

Oshima et al. used plaque hybridization to screen a phage lambda gt11 cDNA library for the gene encoding human placental beta-glucuronidase. Oshima et al., Proceedings of the National Academy of Sciences, U.S.A. 84:685-689 (1987). The identity of isolated cDNA clones was verified by immunoprecipitation of the protein expressed by COS-7 cells transfected with cloned inserts using the SV40 late promoter.

Transient expression in mammalian cells has been employed as a means of confirming the identity of genes previously isolated by other screening methods. Gerald et al., Journal of General Virology 67:2695-2703 (1986). Mackenzie, Journal of Biological Chemistry 261:14112-14117 (1986); Seif et al., Gene 43:1111-1121 (1986); Orkin et al., Molecular and Cellular Biology 5(4):762-767 (1985). These methods often are inefficient and tedious and require multiple rounds of screening to identify full-length or overlapping clones. Prior screening methods based upon expression of fusion proteins are inefficient and require large quantities of monoclonal antibodies. Such drawbacks are compounded by use of inefficient expression vectors, which result in protein expression levels that are inadequate to enable efficient selection.

Summary of the Invention

The present invention relates to a powerful new method for cloning cDNA encoding cell surface antigens, to a method of constructing cDNA libraries, to high efficiency expression vectors particularly suited for high level expression in eukaryotic host cells, and to the isolated nucleotide sequences and their encoded products.

5 The highly efficient cloning technique of the present invention is based upon transient expression of antigen in eukaryotic cells and physical selection of cells expressing the antigen by adhesion to an antibody-coated substrate, such as a culture dish. The methods of the present invention are useful for the isolation and molecular cloning of any protein which can be expressed and transported to the cell surface membrane of a eukaryotic cell.

10 The method for cloning cDNA encoding a cell surface antigen of the present invention comprises preparing a cDNA library; introducing this cDNA library into eukaryotic mammalian cells, preferably tissue culture cells; culturing these cells under conditions allowing expression of the cell surface antigen; exposing the cells to a first antibody or antibodies directed against the cell surface antigen, thereby allowing the formation of a cell surface antigen-first antibody complex; subsequently exposing the cells to a substrate coated with a second antibody directed against the first antibody, thereby causing cells expressing the cell surface antigen to adhere to the substrate via the formation of a cell surface antigen-first antibody-second antibody complex; and separating adherent from non-adherent cells.

25 By means of the cloning method of the present invention, isolation and molecular cloning of genes encoding such cell surface antigens as the following have been accomplished: the CD1a, CD1b, CD1c, CD2, CD6, CD7, CD13, CD14, CD16, CD19, CD20, CD22, CD26, CD27, CD28, CD31, CDw32a, CDw32b, CD33, CD34, CD36, CD37, CD38, CD39, CD40, CD43, CD44, CD53, ICAM, LFA-3, FcRIa, FcRIb, TLIa, and Leu8 antigens. The nucleotide sequences of
30 genes cloned by the method of the present invention have been determined and the amino acid sequences of the encoded proteins have been identified. A cloned gene, such as that encoding CD1a, CD1b, CD1c, CD2, CD6, CD7, CD13, CD14, CD16, CD19, CD20, CD22, CD26, CD27, CD28, CD31, CDw32a, CDw32b, CD33, CD34, CD36, CD37,

CD38, CD39, CD40, CD43, CD44, CD53, ICAM, LFA-3, FcRIa, FcRIb, TLiSa, and Leu8, is also the subject of the present invention.

Once the gene encoding an antigen has been cloned according to the method of the present invention, that gene can be expressed in a prokaryotic or a eukaryotic host cell to produce the encoded protein or portion thereof in substantially pure form such as it does not exist in nature. Another aspect of the present invention relates to substantially pure cell surface antigens, particularly: CD1a, CD1b, CD1c, CD2, CD6, CD7, CD13, CD14, CD16, CD19, CD20, CD22, CD26, CD27, CD28, CD31, CDw32a, CDw32b, CD33, CD34, CD36, CD37, CD38, CD39, CD40, CD43, CD44, CD53, ICAM, LFA-3, FcRIa, FcRIb, TLiSa, and Leu8 antigens and their functional analogues and equivalents. The primary amino acid sequences of the CD1a, CD1b, CD2, CD7, CD14, CD16, CD19, CD20, CD22, CD27, CD28, CDw32a, CDw32b, CD33, CD34, CD40, CD44, CD53, ICAM, LFA-3, FcRIa, FcRIb, TLiSa and Leu8 antigens have been determined. The invention thus also relates to the amino acid sequences of those antigens and their functional equivalents and to the nucleotide sequences encoding those antigens.

This invention also relates to high efficiency cDNA expression vectors which allow the generation of very large mammalian expression libraries and yield large amounts of protein in mammalian host cells, resulting in efficient selection. In a particular embodiment of this invention, a cDNA expression vector comprises a suppressor tRNA gene; an SV40 origin; a synthetic transcription unit, comprising a chimeric promoter composed of human cytomegalovirus AD169 immediate early enhancer sequence fused to the HIV LTR -60 to +80 sequences, inserted between the suppressor tRNA gene and the SV40 origin; a polylinker comprising two BstXI sites separated by a replaceable DNA sequence and flanked by XbaI sites; and an SV40 small t antigen splice and early region polyadenylation signals.

A further aspect of the present invention comprises a synthetic transcription unit for use in a cDNA expression vector,

comprising a chimeric promoter composed of human cytomegalovirus AD169 immediate early enhancer sequences fused to HIV LTR -60 to +80 sequences. The small size and particular arrangement of the sequences of the cDNA expression vector of the present invention allow highly efficient replication in host mammalian tissue culture cells, such as COS cells. Moreover, this vector employs a polylinker containing two inverted BstXI sites separated by a short replaceable DNA segment, which allows the use of very efficient oligonucleotide-based cDNA insertion strategy.

In another aspect, the present invention comprises a vector comprising two identical BstXI sites in inverted orientation each with respect to the other, which BstXI sites are separated by a short replaceable DNA fragment. Another aspect of the invention is a polylinker as described above.

A further aspect of the invention relates to an oligonucleotide-based cDNA insertion method, comprising ligating synthetic DNA oligonucleotides to the cDNA segment desired to be inserted into a vector, the synthetic DNA oligonucleotides giving the same terminal sequences as those of the short replaceable DNA fragment of the polylinker of the invention, and inserting the resulting cDNA segment plus synthetic DNA oligonucleotide terminal sequences into the polylinker of the vector, from which the short replaceable DNA fragment previously has been removed.

In preparing cDNA libraries according to the present invention, it has been discovered that many tumors are heavily infiltrated by macrophages and lymphocytes, and thus may be employed as a source of macrophage or lymphocyte transcripts to good effect, instead of tumor cell lines commonly used. In another aspect, then, the present invention relates to the use of tumor cells, particularly human tumor cells, to prepare cDNA libraries for use according to the methods of the present invention.

Another advantage of the powerful selection system of the present invention is that directional insertion of the cDNA is not necessary. The method of the present invention results in library construction efficiencies which are on a par with those described for phage vectors such as lambda gt10 and lambda gt11, with the additional advantage that clones generated according to the methods of the present invention are easier to manipulate.

The immunoselection technique of the present invention allows efficient use of antibodies, which may be monoclonal or polyclonal, in relatively small absolute amounts. The method of the present invention also is quite rapid. Generally, three or fewer cycles of immunoselection and rescue are required to isolate a target cDNA clone. Thus, the method of the present invention also results in the efficient use of labor and materials when cloning genes encoding cell surface antigens. As described above, this method has been employed to successfully clone genes encoding cell surface antigens associated with mammalian T lymphocytes (e.g. antigens CD1a, CD1b, CD1c, CD2, CD6, CD7, CD13, CD14, CD16, CD19, CD20, CD22, CD26, CD27, CD28, CD31, CDw32a, CDw32b, CD33, CD34, CD36, CD37, CD38, CD39, CD40, CD43, CD44, CD53, ICAM, LFA-3, FcRIa, FcRIb, TLIa, and Leu8).

The purified genes and proteins of the present invention are useful for immunodiagnostic and immunotherapeutic applications, including the diagnosis and treatment of immune-mediated infections, diseases, and disorders in animals, including humans. They can also be used to identify, isolate and purify other antibodies and antigens. Such diagnostic and therapeutic uses comprise yet another aspect of the present invention. Moreover, the substantially pure proteins of the present invention may be prepared as medicaments or pharmaceutical compositions for therapeutic administration. The present invention further relates to such medicaments and compositions.

Brief Description of the Drawings

Figure 1. Nucleotide sequence of expression vector piH3

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Nucleotides 1-589 are derived from pMB1 origin (pBR322 ori); nucleotides 590-597 are derived from the SacII linker (ACCGCGT); nucleotides 598-799 are derived from the synthetic tyrosine suppressor tRNA gene (supF gene); nucleotides 800-947 are derived from a remnant of the ASV LTR fragment (PvuII to MluI); nucleotides 948-1500 are derived from the human cytomegalovirus AD169 enhancer; nucleotides 1501-1650 are derived from HIV TATA and tat-responsive elements; nucleotides 1651-1716 are derived from the piLNKAN polylinker (HindIII to Xba); nucleotides 1717-2569 are derived from pSV to splice and poly-Addition signals; nucleotides 2570-2917 are derived from the SV40 origin of replication (PvuII to (HindIII); and nucleotides 2918-2922 are derived from piVX, remnant of R1 site from polylinker.

Figure 2. Nucleotide sequence of the CD2cDNA insert

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Nucleotide numbering is given in parentheses at right, amino acid numbering, left. Locations of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, as well as the predicted transmembrane (TM) sequence. The amino acid sequence is numbered from the projected cleavage site of the secretory signal sequence.

Figure 3. Restriction map of the CDM8 expression vector

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The CDM8 vector includes a deleted version of a mutant polyoma virus early region selected for high efficiency expression in both murine and monkey cells. Substantially all of the human immunodeficiency promoter region has been replaced with the cognate sequences of the human cytomegalovirus immediate early promoter, and by inclusion of a bacteriophage T7 promoter between the eukaryotic promoter and the site of cDNA insertion. Arrows indicate the direction of transcription.

WOP cells transfected with a clone encoding the LFA-3 antigen were detected by indirect immunofluorescence, amplified and sequenced. (A) shows the 874 base pair insert containing an open reading frame of 237 residues originating at a methionine codon, and terminating in a series of hydrophobic residues. Hydrophobic and hydrophilic regions within this open reading frame are shown in (B).

Figure 5. Restriction Map of the p
The direction of transcription is indicated by an arrow.
Restriction endonuclease sites flanking the BstXI cloning sites
are shown.

Figures 6. Nucleotide sequence of the
There are 7 segments. Residues 1-587 are from the pBR322 origin of replication, 588-1182 from the M13 origin, 1183-1384 from the supF gene, 1385-2238 are from the chimeric cytomegalovirus/human immunodeficiency virus promoter, 2239-2647 are from the replaceable fragment, 2648-3547 from plasmid pSV2 (splice and polyadenylation signals), and 3548-3900 from the SV40 virus origin.

Figures 7. Nucleotide sequence of the
Nucleotide numbering is given in parentheses at right, amino acid numbering, center and left. Location of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, as well as the predicted transmembrane (TM) sequence. The amino acid sequence is numbered from the projected cleavage site of the secretory signal sequence.

Figures 8. Nucleotide sequence of the cDNA
Nucleotide numbering is given in parentheses at right. Splice donor and acceptor sites indicated by (/). The location of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, the potential fatty acid esterification site is denoted (*), and the predicted

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A12
transmembrane domain (TM) is underlined. Nucleotide sequences potentially involved in hairpin formation are denoted by (.). The presumed polyadenylation signal is underlined.

Figures 9. Nucleotide sequence of the CDw32 cDNA

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A13
Nucleotide number is given in the parenthesis at right, amino acid numbering, center and left. Locations of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, as well as the predicted transmembrane (TM) sequence. The amino acid sequence is numbered from the projected cleavage site of the secretory signal sequence. Cysteine residues are underscored with asterisks.

Figures 10. Sequence of the CD20.4 cDNA

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A14
A. The sites of potential N-linked glycosylation are denoted by the symbol -CHO-; the hydrophobic regions are underscored. The site of the poly(A)* tail in clone CD20.6 is denoted by an asterisk.

B. Hydrophobicity profile of the amino acid sequence in A.

Figures 11. Sequence of ICAM-1

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A15
Complete nucleotide sequence of ICAM-1 cDNA insert and predicted protein sequence. Nucleotide numbering is at left, amino acid numbering, center. The RGE motif at position 128 is underlined, the potential N-linked glycosylation sites are indicated by -CHO- and the transmembrane domain by -TM-. The amino acid sequence is numbered from the projected cleavage site of the signal peptide. Sequencing was by dideoxy-chain termination (Sanger, F., et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)), using a combination of subclones, and specific oligonucleotides.

Figures 12. Nucleotide sequence of CD19

Figures 13. Nucleotide sequence of CD20

Figure 15. Nucleotide sequence of CDw32a

Figures 16. Nucleotide sequence of CDw32b

Figure 17. Nucleotide sequence of CD40

Detailed Description of the Invention

5 This invention relates to a novel method for cloning cDNA encoding a cell surface antigen and to a method of constructing cDNA libraries. It also relates to particular cDNA expression vectors and components thereof, nucleotide sequences or genes isolated by the method, substantially pure cell surface antigens encoded by the cDNA segments, and methods of using the isolated nucleotide sequences and encoded products.

10 In the following description, reference will be made to various methodologies known to those of skill in the art of recombinant genetics. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties. Standard reference works setting forth the general principles of recombinant DNA technology include Darnell, J.E. et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publisher, New York, N.Y. (1985); Old, R.W. et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, Berkeley, CA (1981); and Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1982).

25 By "cloning" is meant the use of in vitro recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which

it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by art-recognized methods described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, *supra*. Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines. More preferred are the human tumor cell line HPB-ALL and the human lymphoblastoid cell line JY. Alternatively, RNA can be isolated from a tumor cell, derived from an animal tumor, and preferably from a human tumor. Thus, a library may be prepared from, for example, a human adrenal tumor, but any tumor may be used.

The immunoselection cloning method of the present invention comprises the preparation of a cDNA library by extracting total RNA including a particular gene from a cell, synthesizing a series of complementary double-stranded cDNA fragments from the RNA and introducing these cDNA fragments into mammalian cells in tissue culture. The mammalian cells are maintained under conditions which allow them to express the protein (i.e. the cell surface antigen). The resulting cells are exposed to a first antibody or pool (group) of antibodies directed against the cell surface antigen. This results in formation of a cell surface antigen-first antibody complex. The complexes are exposed to a substrate to which is coated or bound a second antibody directed against the first antibody. Cells expressing the cell surface

antigen adhere to the substrate (because of formation of a cell surface antigen-first antibody-second antibody complex). Adherent cells are separated from non-adherent cells.

Isolation of total RNA

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The guanidium thiocyanate/CsCl method of isolating total RNA is preferred. More preferred is a guanidium thiocyanate/LiCl variant of the GuSCN/CsCl method, which has added capacity and speed. Briefly, for each ml of mix desired, 0.5g GuSCN are dissolved in 0.58 ml of 25% LiCl (stock filtered through 0.45 micron filter) and 20 ul of mercaptoethanol is added. Cells are spun out and the pellet is dispersed on walls by flicking, add 1 ml of solution to up to 5×10^7 cells. The resulting combination is sheared by polytron until nonviscous. For small scale preps (less than 10^8 cells) layer 2 ml of sheared mix on 1.5 ml of 5.7M CsCl (RNase free; 1.26g CsCl added to every ml 10 mM EDTA pH 8), overlay with RNase-free water and spin SW55 50k rpm 2h. For large scale preps, layer 25 ml on 12 ml CsCl in a SW28 tube, overlay, and spin 24k rpm 8h. Aspirate contents carefully with a sterile pasteur pipet connected to a vacuum flask. Once past the CsCl interface, scratch a band around the tube with the pipet tip to prevent the layer on the wall of the tube from creeping down. The remaining CsCl solution is aspirated. The pellets are taken up in water (do not try to redissolve). 1/10 vol. NaOAc and 3 vol. EtOH are added and the resulting combination is spun. If necessary, the pellet is resuspended in water (e.g., at 70°). Adjust concentration to 1 mg/ml and freeze. Small RNA (e.g. 5S) does not come down. For small amounts of cells, scale down volumes and overlay GuSCN with RNase-free water on gradient (precipitation is inefficient when RNA is dilute).

Preparation of poly A⁺ RNA

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Next, polyA⁺ RNA may be prepared, preferably by the oligo dT selection method. Briefly, a disposable polypropylene column is

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prepared by washing with 5M NaOH and then rinsing with RNase-free water. For each milligram total RNA about 0.3 ml (final packed bed) oligo dT cellulose is used. Oligo dT cellulose is prepared by resuspending about 0.5 ml of dry powder in 1 ml of 0.1M NaOH and transferring it into the column, or by percolating 0.1 NaOH through a previously used column (columns can be reused many times). This is washed with several column volumes of RNase-free water, until pH is neutral, and rinsed with 2-3 ml of loading buffer. The column bed is then removed into a sterile 15 ml tube using 4-6 ml of loading buffer. The total RNA to 70°C for 2-3 min., LiCl from RNase-free stock is added (to 0.5M), and combined with oligo dT cellulose in a 15 ml tube. This is followed by vortexing or agitation for 10 min. The result is poured into a column and washed with 3 ml loading buffer and then 3 ml of middle wash buffer. mRNA is eluted directly into an SW55 tube with 1.5 ml of 2 mM EDTA, 0.1% SDS; the first two or three drops are discarded.

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Eluted mRNA is precipitated by adding 1/10 vol. 3M NaOAc and filling the tube with EtOH. This is then mixed, chilled for 30 minutes at -20°C, and spun at 50k rpm at 5°C for 30 min. The EtOH is poured off and the tube is air dried. The mRNA pellet is resuspended in 50-100 ul of RNase-free water. Approximately 5 ul is melted at 70° in MOPS/EDTA/formaldehyde and run on an RNase-free 1% agarose gel to check quality.

25 cDNA Synthesis

From this, cDNA is synthesized. A preferred method of cDNA synthesis is a variant of that described by Gubler and Hoffman (Gene, 25:263-269 (1982)). This is carried out as follows:

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a. First Strand. 4 ug of mRNA and heated to about 100°C in a microfuge tube for 30 seconds and quenched on ice. The volume is adjusted to 70 ul with RNase-free water. The following are added: 20 ul of RT1 buffer, 2 ul of RNase inhibitor (Boehringer 36 u/ul), 1 ul of 5 ug/ul of oligo dT (Col-

laborative Research), 2.5 ul of 20 mM dXTP's (ultrapure), 1 ul of 1 M DTT and 4 ul of RT-LX (Life Science, 24 u/ul). The resulting combination is incubated at 42°C for 40 min. It is heated to inactivate (70°C 10 min).

5 b. Second Strand. 320 ul of RNase free water, 80 ul of RT2 buffer, 5 ul of DNA Polymerase I (Boehringer, 5 U/ul), 2 ul RNase H (BRL 2 u/ul). Incubate at 15°C for 1 hr and 22°C for 1 hr. Add 20 ul of 0.5M EDTA pH 8.0, phenol extract and EtOH precipitate by adding NaCl to 0.5M, linear polyacrylamide (carrier) to 20 ug/ml, and filling tube with EtOH. Spin 2-3 minutes in microfuge, remove, vortex to dislodge precipitate high up on wall of tube, and respin 1 minute.

10 c. Adaptors. Resuspend precipitated cDNA in 240 ul of TE (10/1). Add 30 ul of 10x low salt buffer, 30 ul of 10X low salt buffer, 30 ul of 10X ligation additions, 3 ul (2.4ug) of kinased 12-mer adaptor, 2 ul (1.6ug) of kinased 8-mer adaptor, and 1 ul of T4 DNA ligase (BioLabs, 400 u/ul, or Boehringer, 1 Weiss unit/ml). Incubate at 15°C overnight. Phenol extract and EtOH precipitate as above (no extra carrier now needed), and resuspend in 100 ul of TE.

Use of cDNA fragments in expression vectors

For use with the BstXI-based cDNA expression vectors of the invention (see infra), oligonucleotide segments containing terminal sequences corresponding to BstXI sites on the vectors are ligated to the cDNA fragment desired to be inserted. The resulting fragments are pooled by fractionation. A preferred method is as follows:

30 Prepare a 20% KOA, 2 mM EDTA, 1 ug/ml EthBr solution and a 5% KOAc, 2 mM EDTA, 1 ug/ml EthBr solution. Add 2.6 ml of 20% KOAc solution to back chamber of a small gradient maker. Remove air bubble from tube connecting the two chambers by allowing solution to flow into the front chamber and then tilt back.

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Close passage between chambers, and add 2.5 ml. of the 5% solution to the front chamber. If there is liquid in the tubing from a previous run, allow the 5% solution to run just to the end of the tubing, and then return to chamber. Place the apparatus on a stirplate, set the stir bar moving as fast as possible, open the stopcock connecting the two chambers and then open the front stopcock. Fill a polyallomer SW55 tube from the bottom with the KOAc solution. Overlay the gradient with 100 ul of cDNA solution. Prepare a balance tube and spin the gradient for 3 hrs at 50k rpm at 22°C. To collect fractions from the gradient, pierce the SW55 tube with a butterfly infusion set (with the luer hub clipped off) close to the bottom of the tube and collect three 0.5 ml fractions and then 6 0.25 ml fractions into microfuge tubes (about 22 and 11 drops respectively). EtOH precipitate the fractions by adding linear polyacrylamide to 20 ug/ml and filling the tube to the top with EtOH. After cooling tubes, spin them in a microfuge for 3 min. Vortex and respin 1 min. Rinse pellets with 70% EtOH (respin). Do not dry to completion. Resuspend each 0.25 ml fraction in 10 ul of TE. Run 1 ul on a 1% agarose minigel. Pool the first three fractions, and those of the last six which contain no material smaller than 1 kb.

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Suppressor tRNA plasmids may be propagated by known methods. In a preferred method according to the present invention, sup^F plasmids can be selected in nonsuppressing hosts containing a second plasmid, p3, which contains amber mutated ampicillin and tetracycline drug resistance elements (Seed, 1983). The p3 plasmid is derived from PR1, is 57 kb in length, and is a stably maintained, single copy episome. The ampicillin resistance of this plasmid reverts at a high rate, so that amp^r plasmids usually cannot be used in p3-containing strains. Selection for tet resistance alone is almost as good as selection for amp^r+tet resistance. However, spontaneous appearance of chromosomal suppressor tRNA mutations presents an unavoidable background (frequency about 10⁻⁹) in this system. Colonies arising from spontaneous suppressor mutations are usually bigger than colonies

arising from plasmid transformation. Suppressor plasmids typically are selected for in LB medium containing amp at 12.5 ug/ml and tet at 7.5 ug/ml. For large plasmid preps, M9 casamino acids medium containing glycerol (0.8%) may be used as a carbon source, and the bacteria grown to saturation.

Vector DNA may be isolated by known methods. The following method is preferred for plasmid from 1 liter of saturated cells:

Spin down cells in 1 liter J6 bottles, 4.2k rpm, 25 minutes. Resuspend in 40 ml 10 mM EDTA pH 8 (Thump on soft surface). Add 80 ml 0.2M NaOH, 1% SDS, swirl until clearish, viscous. Add 40 ml 5M KOAc, pH 4.7 (2.5M KOAc, 2.5M HOAc) shake semi-vigorously (until lumps are 2-3 mm in size). Spin (same bottle) 4.2K rpm, 5 min. Pour supernatant through cheesecloth into 250 ml bottle. Fill bottle with isopropyl alcohol. Spin J6, 4.2k rpm, 5 min. Drain bottle, rinse gently with 70% EtOH (avoid fragmenting the pellet). Invert bottle, and remove traces of EtOH with Kimwipe. Resuspend in 3.5 ml Tris base/EDTA 20mM/10mM. Add 3.75 ml of resuspended pellet to 4.5g CsCl. Add 0.75 ml 10 mg/ml ethidium bromide, mix. Fill VTi80 tubes with solution. Run at a speed of 80 rpm for 2.5 hours or longer. Extract bands by visible light with 1 ml syringe and 20 gauge or lower needle. Cut top off tube, insert the needle upwards into the tube at an angle of about 30° with respect to the tube, (i.e., as shallowly possible) at a position about 3 mm beneath the band, with the bevel of the needle up. After the band is removed, pour tube contents into bleach. Deposit extracted bands in 13 ml Sarstedt tube. Fill tube to top with n-butanol saturated with 1M NaCl, extract. If a very large quantity of DNA is obtained, reextract. Aspirate butanol into trap containing 5M NaOH (to destroy ethidium). Add about equal volume 1M ammonium acetate to DNA (squirt bottle). Add about 2 volumes 95% ethanol (squirt bottle). Spin 10K rpm, 5 min. J2-21. Rinse pellet carefully with 70% ethanol. Dry with swab, or lyophilizer.

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The vector may be prepared for cloning by known methods. A preferred method begins with cutting 20 ug of vector in a 200 ul reaction with 100 units of BstXI (New York Biolabs), cutting at 50°C overnight in a well-thermostatted water bath (i.e., circulating water bath). Prepare 2 KOAc 5-20% gradients in SW55 tubes as described above. Add 100 ul of the digested vector to each tube and run for 3 hrs, 50K rpm at 22°C. Examine the tube under 300 nm UV light. The desired band will have migrated 2/3 of the length of the tube. Forward trailing of the band means the gradient is overloaded. Remove the band with a 1 ml syringe and 20 gauge needle. Add linear polyacrylamide and precipitate the plasmid by adding 3 volumes of EtOH. Resuspend in 50 ul of TE. Set up ligations using a constant amount of vector and increasing amounts of cDNAs. On the basis of these trial ligations, set up large scale ligation, which can be accomplished by known methods. Usually the entire cDNA prep requires 1-2 ug of cut vector.

Adaptors may be prepared by known methods, but it is preferred to resuspend crude adaptors at a concentration of 1 ug/ul, add $MgSO_4$ to 10 mM, and precipitate by adding 5 volumes of EtOH. Rinse with 70% EtOH and resuspend in TE at a concentration of 1 ug/ul. To kinase take 25 ul of resuspended adaptors, add 3 ul of 10X kinasing buffer and 20 units of kinase; incubate 37°C overnight.

Preparation of buffers mentioned in the above description of preferred methods according to the present invention will be evident to those of skill. For convenience, preferred buffer compositions are as follows:

Loading Buffer: 0.5 M LiCl, 10 mM Tris pH 7.5, 1mM EDTA 0.1% SDS.

Middle Wash Buffer: 0.15 M LiCl, 10 mM Tris pH 7.5, 1mM EDTA 0.1% SDS.

Rt1 Buffer: 0.25 M Tris pH 8.8 (8.2 at 42°),
0.25 M KCl, 30 mM $MgCl_2$.

RT2 Buffer: 0.1 M Tris pH 7.5, 25 mM $MgCl_2$, 0.5

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- 10X Low Salt: M KCl, 0.25 mg/ml BSA, 50 mM DTT.
60 mM Tris pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 2.5 mg/ml BSA, 70 mM Me.
- 5 10X Ligation Additions: 1 mM ATP, 20 mM DTT, 1 mg/ml BSA, 10 mM spermidine.
- 10X Kinasing Buffer: 0.5 M Tris pH 7.5, 10 mM ATP, 20mM DTT, 10 mM spermidine, 1 mg/ml BSA 100 mM MgCl₂.

10 By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of replicating in a host independently of the host's chromosome, after additional sequences of DNA have been incorporated into the autonomous element's genome. Such DNA expression vectors include bacterial plasmids and phages.

25 Preferred for the purposes of the present invention, however, are viral vectors, such as those derived from simian virus strain 40 (SV40). SV40 is a papovavirus having a molecular weight of 28 Mdal, and containing a circular double-stranded DNA molecule having a molecular weight of 3 Mdal, which comprises the entire genome of the virus. The entire nucleotide sequence of this single, small, covalently closed circular DNA molecule has been determined. Fiers et al., Nature 273:113-120 (1978); Reddy et al., Science 200:494-502 (1978). The viral DNA of SV40 may be obtained in large quantities, and the genomic regions responsible for various viral functions have been accurately located with respect to a detailed physical map of the DNA.

30 Fiers et al., supra; Reddy et al., supra. The viral genome of SV40 can multiply vegetatively or as an integral part of cellular chromosomes, and a wealth of information exists on the replication and expression of this genome.

Also preferred for the purposes of the present invention is a single-stranded bacteriophage cloning vehicle, designated M13, having a closed circular DNA genome of approximately 6.5 kb. An advantage of utilizing M13 as a cloning vehicle is that the phage particles released from infected cells contain single-stranded DNA homologous to only one of the two complementary strands of the cloned DNA, which therefore can be used as a template for DNA sequencing analysis.

Even more preferred for the purposes of the present invention are the expression vectors designated piH3, piH3M, and CDM8, deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852. piH3 was deposited at the ATCC on February 24, 1988, and has accession number ATCC 67634. piH3M was deposited at the ATCC on February 24, 1988, and has accession number ATCC 67633. CDM8 was deposited at the ATCC on February 24, 1988, and has accession number ATCC 67635.

By "tissue culture" is meant the maintenance or growth of animal tissue cells in vitro so as to allow further differentiation and preservation of cell architecture or function or both. "Primary tissue cells" are those taken directly from a population consisting of cells of the same kind performing the same function in an organism. Treating such tissue cells with the proteolytic enzyme trypsin, for example, dissociates them into individual primary tissue cells that grow well when seeded onto culture plates at high densities. Cell cultures arising from multiplication of primary cells in tissue culture are called "secondary cell cultures." Most secondary cells divide a finite number of times and then die. A few secondary cells, however, may pass through this "crisis period", after which they are able to multiply indefinitely to form a continuous "cell line." Cell lines often will contain extra chromosomes, and usually are abnormal in other respects as well. The immortality of these cells is a feature shared in common with cancer cells.

Preferred cell lines for use as tissue culture cells according to the present invention include the monkey kidney cell line, designated "COS." COS cells are those that have been transformed by SV40 DNA containing a functional early gene region but a defective origin of viral DNA replication. COS cell clone M6 is particularly preferred for use according to the method of the invention. Also preferred for the purposes of the present invention are murine "WOP" cells, which are NIH 3T3 cells transfected with polyoma origin deletion DNA. cDNA may be introduced into the host tissue culture cells of the present invention by any methods known to those of skill. Transfection may be accomplished by, for example, protoplast fusion, by spheroplast fusion, or by the DEAE dextran method (Sussman et al., Cell. Biol. 4:1641-1643 (1984)).

If spheroplast fusion is employed, a preferred method is the following variant based on Sandri-Goldrin et al., Mol. Cell Bio. 1:743-752 (1981). Briefly, for example, a set of six fusions requires 100 ml of cells in broth. Grow cells containing amplifiable plasmid to OD 600=0.5 in LB. Add spectinomycin to 100 ug/ml (or chloramphenicol to 150 ug/ml). Continue incubation at 37°C with shaking for 10-16 hours. (Cells begin to lyse with prolonged incubation in spectinomycin or chloramphenicol medium). Spin down 100 ml of culture (JA14/GSA rotor, 250 ml bottle) 5 min. at 10,000 rpm. Drain well, resuspend pellet in bottle with 5 ml cold 20% sucrose, 50 mM Tris-HCL pH 8.0. Incubate on ice 5 min. Add 2 ml cold 0.25M EDTA pH 8.0, incubate 5 min. at 37°C (waterbath). Place on ice, check percent conversion to spheroplasts by microscopy. In flow hood, slowly add 20 ml of cold DME/10% sucrose/10 mM MgCl₂ (dropwise, ca. 2 drops per second). Remove media from cells plated the day before in 6 cm dishes (50% confluent). Add 5 ml of spheroplast suspension to each dish. Place dishes on top of tube carriers in swinging bucket centrifuge. Up to 6 dishes can be comfortably prepared at once. Dishes can be stacked on top of each other, but 3 in a stack is not advisable as the spheroplast layer on the top dish is often torn or detached after centrifugation. Spin at 1000xg

10 min. Force is calculated on the basis of the radius to the bottom plate. Aspirate fluid from dishes carefully. Pipet 1.5-2 ml 50% (w/w) PEG 1450 (or PEG 1000)/50% DME (no serum) into the center of the dish. If necessary, sweep the pipet tip around to ensure that the PEG spreads evenly and radially across the whole dish. After PEG has been added to the last dish, prop all of the dishes up on their lids so that the PEG solution collects at the bottom. Aspirate the PEG. The thin layer of PEG that remains on the cells is sufficient to promote fusion; the layer remaining is easier to wash off, and better cell viability can be obtained, than if the bulk of the PEG is left behind. After 90 to 120 seconds (PEG 1000) or 120 to 150 seconds (PEG 1450) of contact with the PEG solution, pipet 1.5 ml of DME (no serum) into the center of the dish. The PEG layer will be swept radially by the DME. Tilt the dishes and aspirate. Repeat the DME wash. Add 3 ml of DME/10% serum containing 15 ug/ml gentamicin sulfate. Incubate 4-6 hours in incubator. Remove media and remaining bacterial suspension, add more media and incubate 2-3 days. Extensive washing of the cell layer to remove PEG tends to remove many of the cells without any substantial benefit. If the cells are allowed to sit in the second DME wash for a few minutes, most of the spheroplast layer will come up spontaneously; however it is preferred to wash briefly and allow the layer to come off in the complete medium at 37°C.

The PEG solution can be conveniently prepared by melting a fresh bottle of PEG at 60°C and pouring approximate 50 ml aliquots by means of a 50 ml centrifuge tube into preweighed bottles. The aliquoted PEG is stored at 5°C in the dark. To make up a fresh bottle, weigh the aliquot, remelt, and add an equal volume of DME (no serum). Adjust the pH with 7.5% Na bicarbonate solution if necessary, and filter sterilize. The resulting PEG solution may be stored up to 3 months at room temperature without detectable adverse consequence.

Transfected host cells will be cultured according to the invention in order to accomplish expression of the protein

5 encoded by the cDNA clone, and to increase the absolute numbers
of cells available for subsequent immunoselection. Those skilled
in the art will know of appropriate methods and media for this
purpose, taking into account the cell type and other variables
routinely considered. COS cells, for example, may be cultured
in Dulbecco's modified Eagle's medium (DME) supplemented with 10%
calf serum and gentamycin sulfate. Transient expression of
transfected cells normally can be expected between 48 and 72
hours posttransfection. However, this time period may vary
10 depending upon the type or strain of host cell used and the cell
culture conditions, as will be apparent to those of ordinary
skill.

Immunoprecipitation, blotting, and cDNA sequencing of genes
cloned according to the methods of the present invention may be
carried out by any convenient methods known to those of skill.
For example, the immunoprecipitation protocol of Clark et al.,
Leukocyte Typing II, Vol. II, pp. 155-167 (1986), is preferred.
Southern, Northern, or other blot analysis methods known to those
of skill may be employed, using hybridization probes prepared by
known methods, such as that of Hu et al. (Gene 18:271-277
(1982)). cDNA sequencing also may be accomplished by known
methods, including the dideoxynucleotide method of Sanger et al.,
Proc. Natl. Acad. Sci. (USA) 74:5463-5467 (1977).

25 The antibodies used according to the present invention may
be polyclonal or monoclonal. These may be used singly, or in
conjunction with other polyclonal or monoclonal antibodies to
effect immunoselection of cells expressing the desired antigen
or antigens by the methods of the present invention. Methods of
preparing antibodies or fragments thereof for use according to
30 the present invention are known to those of skill.

Standard reference works setting forth general principles
of immunology include Klein, J., Immunology: The Science of
Self-Nonself Discrimination, John Wiley & Sons, publisher, New
York (1982); Kennett, R., et al., eds., Monoclonal Antibodies,

Hybridoma: A New Dimension in Biological Analyses, Plenum Press,
publisher, New York (1980); Campbell, A., "Monoclonal Antibody
Technology" in Burden, R., et al., eds., Laboratory Techniques
in Biochemistry and Molecular Biology, Vol. 13, Elsevier,
publisher, Amsterdam (1984).

The term "antibody" is meant to include the intact molecule
as well as fragments thereof, such as, for example, Fab and
F(ab)'₂ fragments, which also are able to bind to antigen.
Polyclonal antibody preparations may be derived directly from the
blood of the desired animal species after immunization with the
antigen of interest, or a fragment thereof, using any of the
standard protocols known to those of ordinary skill. Similarly,
monoclonal antibodies may be prepared using known methods (Kohler
et al., Eur. J. Immunol. 6:292 (1976)). Use of monoclonal
antibodies is preferred for the purposes of the present
invention.

For the purposes of immunoselection according to the present
invention, the tissue culture host cells which have been exposed
to antibodies directed against the target cell surface antigen
are separated from host cells which do not express the target
antigen by distributing the cells onto a substrate coated with
antibody directed against the antigen. This
technique, termed "panning," will be known to those of skill, and
is described, for example, by Mage et al., J. Immunol. Meth.
15:47-56 (1977), and Wysocki and Sato, Proc. Natl. Acad. Sci.
(USA) 75:2844-2848 (1978).

Panning according to the methods of the present invention
may be carried out as follows:

a. Antibody-coated dishes. Bacteriological 60 mm
plates, Falcon 1007 or equivalent, or 10 cm dishes such as Fisher
8-757-12 may be used. Sheep anti-mouse affinity purified
antibody (from, for example, Cooper BioMedical (Cappell)) is
diluted to 10 ug/ml in 50 mM Tris HCl, pH 9.5. Add 3 ml per 6

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cm dish, or 10 ml per 10 cm dish. Let sit ca. 1.5 hrs., remove to next dish 1.5 hrs., then to 3rd dish. Wash plates 3x with 0.15 NaCl (a wash bottle is convenient for this), incubate with 3 ml 1 mg/ml BSA in PBS overnight, aspirate and freeze.

5 b. Panning. Cells will be in 60 mm dishes. Aspirate medium from dish, add 2 ml PBS/0.5 mM EDTA/0.02% azide and incubate dishes at 37°C for 30 min. to detach cells from dish. Triturate cells vigorously with short pasteur pipet, and collect cells from each dish in a centrifuge tube. Spin 4 min. setting 2.5 (200 x g) (takes 5 min). Resuspend cells in 0.5-1.0 ml PBS/EDTA/azide/5% FBS and add antibodies. Incubate at least 30 min. on ice. Add an equal volume of PBS/EDTA/azide, layer carefully on 3 ml PBS/EDTA/azide/2% Ficoll, and spin 4 min. at setting 2.5. Aspirate supernatant in one smooth movement. Take up cells in 0.5 ml PBS/EDTA/azide and add aliquots to antibody-coated dishes containing 3 ml PBS/EDTA/azide/5% FBS by pipetting through 100 micron Nylon mesh (Tetko). Add cells from at most two 60 mm dishes to one 60 mm antibody-coated plate. Let sit at room temperature 1-3 hours. Remove excess cells not adhering to dish by gentle washing with PBS/5% serum or with medium. 2 or 3 washes of 3 ml are usually sufficient.

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c. Hirt Supernatant. A preferred variant of the method of Hirt, J. Molec. Biol. 26:365-369 (1967), is as follows:
25 Add 0.4 ml 0.6% SDS, 10 mM EDTA to panned plate. Let sit 20 minutes (can be as little as 1 min. if there are practically no cells on the plate). Pipet viscous mixture into microfuge tube. Add 0.1 ml 5M NaCl, mix, put on ice at least 5 hrs. Keeping the mixture as cold as possible seems to improve the quality of the Hirt. Spin 4 min., remove supernatant carefully, phenol extract
30 (twice if the first interface is not clean), add 10 ug linear polyacrylamide (or other carrier), fill tube to top with EtOH, precipitate, and resuspend in 0.1 ml. Add 3 volumes EtOH/NaOAc, reprecipitate and resuspend in 0.1 ml. Transform into MC1061/p3, preferably using the high efficiency protocol hereinafter
35 described. If the DNA volume exceeds 2% of the competent cell

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aliquot, the transformation efficiency will suffer. 5% gives the same number of colonies as 2.5% (efficiency is halved).

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It is preferred for this aspect of the present invention to use "blockers" in the incubation medium. Blockers assure that non-specific proteins, proteases, or antibodies present do not cross-link with or destroy the antibodies present on the substrate or on the host cell surface, to yield false positive or false negative results. Selection of blockers can substantially improve the specificity of the immunoselection step of the present invention. A number of non-specific monoclonal antibodies, for example, of the same class or subclass (isotype) as those used in the immunoselection step (e.g., IgG₁, IgG_{2A}, IgGm, etc.) can be used as blockers. Blocker concentration (normally 1-100 ug/ul) is important to maintain the proper sensitivity yet inhibit unwanted interference. Those of skill also will recognize that the buffer system used for incubation may be selected to optimize blocking action and decrease non-specific binding.

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A population of cells to be panned for those expressing the target cell surface antigen is first detached from its cell culture dish (harvested) without trypsin. The cells then are exposed to a first antibody, which may be polyclonal or monoclonal, directed against the antigen of interest or against a family of related antigens. At this initial stage, a single antibody or a group of antibodies may be used, the choice depending upon the nature of the target antigen, its anticipated frequency, and other variables that will be apparent to those of skill. Target antigens expressed on the surfaces of host cells will form an antigen-antibody complex.

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The cells subsequently are placed in close apposition to a substrate, such as a culture dish, filter disc, or the like, which previously has been coated with a second antibody or group of antibodies. This second antibody will be directed against the first antibody, and its choice will be a matter of ordinary skill

dictated by, for example, the animal in which the first antibody was raised. For example, if the first antibody was raised in mice, the second antibody might be directed against mouse immunoglobulins, raised in goats or sheep. Cells expressing the target antigen will adhere to the substrate via the complex formed between the antigen, the first antibody, and the second antibody. Adherent cells then may be separated from nonadherent cells by washing. DNA encoding the target antigen is prepared from adherent cells by known methods, such as that of Hirt, J. Molec. Biol. 26:365-369 (1967). This DNA may be transformed into E. coli or other suitable host cells for further rounds of fusion and selection, to achieve the desired degree of enrichment.

In the usual case, the initial rounds of immunoselection will employ a panel of first antibodies directed against an epitope or group of epitopes common to the family of antigens to which the target antigen belongs. This will be sufficient to narrow the number of clones for future rounds quite significantly. Two such rounds usually will be found adequate, but the number of rounds may vary as mentioned above. Thereafter, a single round of selection may be performed employing a single first antibody or a group of first antibodies recognizing only the target antigen.

By "substrate" is meant a solid surface to which antibodies may be bound for immunoselection according to the present invention. Known suitable substrates include glass, polystyrene, polypropylene, dextran, nylon, and other materials. Tubes, beads, microtiter plates, bacteriological culture dishes, and the like formed from or coated with such materials may be used. Antibodies may be covalently or physically bound to the substrate by known techniques, such as covalent bonding via an amide or ester linkage, or by absorption. Those skilled in the art will know many other suitable substrates and methods for immobilizing antibodies thereupon, or will be able to ascertain such substrates and methods using no more than routine experimentation.

5 The choice of host tissue culture cells for use according to the present invention preferably should be such as to avoid the situation in which the antibodies used for panning recognize determinants on untransfected cells. Thus, while COS cells are preferred for transient expression of certain surface antigens, more preferred are murine WOP cells. Of the latter, WOP 3027 cells are even more preferred. WOP cells allow virtually all antibodies to be used, since cross-reactions between murine antibodies and murine cell surface determinants are rare.

10 The insert size of the recombinant DNA molecule should be chosen to maximize the likelihood of obtaining an entire coding sequence. Those of skill will know various methods by which a preliminary determination of optimal insert size for a given gene may be determined.

Vector construction and cDNA insertion

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25 Vectors suitable for expression of cDNA in mammalian tissue culture cells may be constructed by known methods. Preferred for the purposes of the present invention is an expression vector containing the SV40 origin. The vector may contain a naturally derived or synthetic transcription origin, and the SV40 early region promoter. Even more preferred is a chimeric promoter composed of human cytomegalovirus immediate early enhancer sequences. Various "enhancer sequences" also may be used with SV40 vectors. These are described, for example, by Banerji et al., Cell 27:299-308 (1981); Levinson et al., Nature 295:568-572 (1982); and Conrad et al., Mol. Cell. Biol. 2:949-965 (1982).

30 ~~Insertion of cDNA into the vectors of the present invention can occur, for example, by homopolymeric tailing with terminal transferase. However, homopolymeric tracts located 5' to cDNA inserts may inhibit in vitro and in vivo expression. Thus, preferred for purposes of the present invention is the use of inverted identical cleavage sites separated by a short replaceable DNA segment. Such inverted identical cleavage sites,~~

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preferably employing the BstXI restriction endonuclease, may be used in parallel with cDNA synthetic oligonucleotides, giving the same terminii as the replaceable segment of the vector. In this manner, the cDNA cannot ligate to itself, but can ligate to the vector. This allows the most efficient use of both cDNA and vector.

Another embodiment of the present invention is the above-described efficient oligonucleotide-based strategy to promote cDNA insertion into the vector. The piH3M vector of the present invention is preferred, and employs the inverted endonuclease sites. This vector may contain an SV40 origin of replication, but a more preferred form contains an M13 origin. This vector, containing the M13 origin, allows high level expression in COS cells of coding sequences placed under its control. Also, the small size and particular arrangement of sequences in the plasmid permit high level replication in COS cells.

By "cell surface antigen" is meant any protein that is transported through the intracellular membrane system to the cell surface. Such antigens normally are anchored to the cell surface membrane through a carboxyl terminal domain containing hydrophobic amino acids that lie in the lipid bilayer of the membrane, and there exert their biological and antigenic effects. Antigens such as those of T-lymphocytes are particularly suited for gene cloning by the method of the present invention. However, cell surface antigens of any cells may be cloned according to the present method. Moreover, proteins not normally expressed on the cell surface may admit of cloning according to the present method by, for example, using fluorescence activated cell sorting (FACS) to enrich for fixed cells expressing intracellular antigens.

By "substantially pure" is meant any antigen of the present invention, or any gene encoding any such antigen, which is essentially free of other antigens or genes, respectively, or of

other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.

By "functional derivative" is meant the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule.

5 A "fragment" of a molecule, such as any of the antigens of the present invention, is meant to refer to any polypeptide subset of the molecule containing a functional domain such as an epitope, a ligand binding site, an extracellular domain or an immunoglobulin domain, which comprises at least about 6 amino acids. A "variant" of such molecules is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

25 A fragment, variant, analog and/or chemical derivative of a subject antigen is said to be a "functional derivative" of the antigen if the amino acid of the former has at least about 80% identity to the sequence of the latter, and if the former has at least about 30% of a biological activity or function of the latter. Increasingly preferred are amino acid identities that increase integrally, i.e., at least about 81%, 82%, etc. identity. Also, increasingly preferred biological activities are those of at least about 40%, 50%, 60%, 70%, 80%, and 90%.

30 A nucleotide sequence is said to be a "functional derivative" of a disclosed nucleotide sequence encoding an

antigen if the former encodes a disclosed antigen or a functional derivative thereof.

Biological activities are those operations, functions or processes which are characteristic of living organisms. Biological activities can also include the reproduction, extension or adaptation of living processes to in vitro or non-natural systems, such as the biological activity exhibited when an antigen or its functional derivative is artificially introduced into a test animal to induce the production of antibodies. An antigen can have one or more biological activities. Biological activities can be detected or measured by methods or assays that are characteristic for that activity. For a functional derivative to have a biological activity substantially the same as that of an antigen, it must have a biological activity of at least about 30% of that of antigen as measured by an assay characteristic for that activity and known to those of skill in the art.

The substantially pure antigens that have been expressed by methods of the present invention may be used in immunodiagnostic assay methods well known to those of skill, including radio-immunoassays (RIAs), enzyme immunoassays (EIAs) and enzyme-linked immunosorbent assays (ELISAs). The substantially pure proteins of the present invention, in soluble form, may be administered alone or in combination with other antigens of the present invention, or with other agents, including lymphokines and monokines or drugs, for the treatment of immune-related diseases and disorders in animals, including humans. As examples of such disorders that may benefit from treatment with the substantially pure proteins of the present invention may be mentioned immune deficiency diseases, diseases of immediate type hypersensitivity, asthma, hypersensitivity pneumonitis, immune-complex disease, vasculitis, systemic lupus erythematosus, rheumatoid arthritis, immunopathogenic renal injury, acute and chronic inflammation, hemolytic anemias, platelet disorders, plasma and other cell neoplasms, amyloidosis, parasitic diseases, multiple sclerosis,

Guillain-Barre syndrome, acute and subacute myopathic paralysis, myasthenia gravis, immune endocrinopathies, and tissue and organ transplant rejection, all as described in Petersdorf et al., eds., Harrison's Principles of Internal Medicine, supra. See also Weir, ed., supra; Boguslaski et al., eds., supra; and Holborow et al., eds., supra.

When used for immunotherapy, the antigens of the present invention may be unlabeled or labeled with a therapeutic agent. Examples of therapeutic agents which can be coupled to the antigens of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

The dose ranges for the administration of the antigens of the present invention are those large enough to produce the desired immunotherapeutic effect, but not so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage employed will vary with the age, condition, sex, and extent of the disease in the patient. Counterindications (if any), immune tolerance and other variables also will affect the proper dosage. Administration may be parenteral, by injection or by gradual perfusion over time. Administration also may be intravenous, intraparenteral, intramuscular, subcutaneous, or intradermal.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions and emulsions. Examples of non-aqueous solvents include propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic and aqueous solutions, emulsions, or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives also

may be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like. Such preparations, and the manner and method of making them, are known and described, for example, in Remington's Pharmaceutical Science, 16th ed., supra.

The antigens of the present invention also may be prepared as medicaments or pharmaceutical compositions comprising the antigens, either alone or in combination with other antigens or other agents such as lymphokines, monokines, and drugs, the medicaments being used for therapy of animal, including human, immune-related indications.

Although the antigens of the present invention may be administered alone, it is preferred that they be administered as a pharmaceutical composition. The compositions of the present invention comprise at least one antigen or its pharmaceutically acceptable salt, together with one or more acceptable carriers and optionally other therapeutic agents. By "acceptable" is meant that the agent or carrier be compatible with other ingredients of the composition and not injurious to the patient. Compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, or parenteral administration. The compositions conveniently may be presented in unit dosage form, and may be prepared by methods well known in the pharmaceutical arts. Such methods include bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers, or both, and shaping the product formed thereby, if required.

Orally administered pharmaceutical compositions according to the present invention may be in any convenient form, including capsules, cachets, or tablets, each containing a predetermined amount of the active ingredient. Powders or granules also are

possible, as well as solution or suspension in aqueous or nonaqueous liquids, or oil-in-water liquid emulsions, or water-in-oil liquid emulsions. The active ingredient also may be presented as a bolus, electuary or paste.

5 Having now described the invention, the same will be more fully understood by reference to the following examples, which are not intended in any way to limit the scope of the invention.

Example I Isolation, Molecular Cloning, and Structure of the Human CD2 Antigen

10 The cDNA expression vector piH3

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A COS cell expression vector was constructed from piSV (Little et al., Mol. Biol. Med. 1:473-488 (1983)) by inserting a synthetic transcription unit between the suppressor tRNA gene and the SV40 origin. The transcription unit consisted of a chimeric promoter composed of human cytomegalovirus AD169 immediately early enhancer sequences fused to the HIV LTR -67 to +80 sequences. Immediately downstream from the LTR +80 sequence was inserted a polylinker containing two BstXI sites separated by a 350bp stuffer; the BstXI sites were flanked by XbaI sites, which could also be used to excise the insert. Downstream from the polylinker were placed the SV40 small t antigen splice and early region polyadenylation signals derived from pSV2. The nucleotide sequence of the vector is shown in Figure 1.

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cDNA library construction

30 RNA was prepared from HPB-ALL cells by the guanidinium thiocyanate/CsCl method, as described above. PolyA⁺ RNA was prepared from total RNA by oligo dT selection. Maniatis et al, Molecular Cloning: A Laboratory Manual, supra. cDNA was synthesized by the method of Gubler and Hoffman (Gene 25:263-269 (1982)). BstXI adaptors were ligated to the cDNA, and the reaction products fractionated by centrifugation through a 5 ml-20% potassium acetate gradient containing 1 mM EDTA for 3 hours at 50k rpm in a SW55 rotor. 0.5 ml fractions were collected manually through a syringe needle or butterfly inserted just
35 above the curve of the tube. Individual fractions were ethanol-

37
precipitated after addition of linear polyacrylamide (Strauss and Varshavsky, Cell 37:889-901 (1984)) to 20 ug/ml. Fractions containing cDNA larger than 700bp were pooled and ligated to gradient purified BstXI digested pIH3 vector.

5/13
10 The ligated DNA was transformed into E. coli MC1061/p3 made competent by the following protocol: The desired strain was streaked out on an LB plate. The next day a single colony was inoculated into 20 ml TYM broth (recipes below) in a 250 ml flask. The cells were grown to midlog phase (OD₆₀₀ about 0.2-0.8), poured into a 21 flask containing 100 ml TYM, and vigorously agitated until cells grew to 0.5-0.9 OD, then diluted again to 500 ml in the same vessel. When the cells grew to OD₆₀₀ 0.6, the flask was placed in ice-water, and shaken gently to assure rapid cooling. When the culture was cool, it was spun at 4.2k rpm for 15 minutes (J6). The supernatant was poured off and the pellet resuspended in about 100 ml cold Tfb I (below) by gentle shaking on ice. Thereafter, it was respun in the same bottle at 4.2k rpm for 8 minutes (J6). The supernatant was poured off and the pellet resuspended in 20 ml cold Tfb II by gentle shaking on ice. 0.1 to 0.5 ml aliquots were placed in prechilled microfuge tubes, frozen in liquid nitrogen, and stored at -70°C. For transformation, an aliquot was removed, thawed at room temperature until just melting, and placed on ice. DNA was added, let sit on ice 15-30 minutes, and incubated at 37°C for 5 minutes (6 minutes for 0.5 ml aliquots). Thereafter the DNA-containing suspensions were diluted 1:10 in LB and grown for 90 minutes before plating or applying antibiotic selection. Alternatively, the heat-pulsed transformation mix was plated directly on antibiotic plates onto which a thin (4-5 ml) layer of antibiotic-free LB agar was poured just before plating.

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35 Media and Buffers: TYM: 2% Bacto-Tryptone, 0.5% Yeast Extract, 0.1M NaCl, 10 mM MgSO₄ (can be added before autoclaving). Tfb I: 30 mM KOAc, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂, 15% (v/v) glycerol. Tfb II: 10 mM Na-MOPS, pH 7.0, 75 mM CaCl₂, 10 mM KCl, 15% glycerol.

Recovery of cDNA clones by panning

Bacteriological culture dishes (Falcon 1007) were prepared for panning by coating with an affinity purified sheep anti-mouse IgG antibody as described by Wysocki and Sato (Proc. Natl. Acad. Sci. USA 75:2844-2848 (1978)), except that dishes were washed with 0.15M NaCl from a wash bottle instead of PBS, and unreacted sites were blocked by overnight incubation in PBS containing 1 mg/ml BSA. Dishes were typically prepared in large batches and stored frozen, after aspiration of the PBS/BSA. In the first round of screening, 24 6 cm dishes of 50% confluent COS cells were transfected by protoplast fusion according to the method of Sandri-Goldrin et al., Mol. Cell Biol. 1:743-752 (1981). 72 hours post fusion the cells were detached by incubation in PBS/1 mM EDTA/.02% sodium azide at 37°C for 30 minutes. The detached cells were pooled, centrifuged, and resuspended in cold PBS/EDTA/5% Fetal Bovine Serum containing monoclonal antibodies, usually as ascites at 1:1000 dilution, but also as commercial reagents at the concentrations suggested by the manufactures. After 1 hour on ice, the cells were diluted with 1:1 with PBS/EDTA/azide and layered on 10 ml of PBS/EDTA/ azide containing 2% Ficoll 400. After centrifugation (400xg, 5 minutes), the supernatant was carefully aspirated, the pellet resuspended in a small amount of PBS/EDTA/5% FBS, and the cells distributed into panning plates containing 3 ml of PBS/EDTA/5% FBS. The plates were then treated essentially as described by Wysocki and Sato, Proc. Natl. Acad. Sci. USA 75:2844-2848 (1978). Episomal DNA was recovered from the adherent cells by the Hirt (J. Mol. Biol. 26:365-269 (1967)) procedure and transformed into MC1061/p3.

Cell lines and cell culture

COS cell clone M6 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and gentamycin sulfate at 15 ug/ml (DME/10% calf serum). Cells were split the day before transfection in 6 cm dishes at approximately 1:8 ratio from stock plates kept as dense as possible without overtly affronting the cells. T cell lines were grown in Iscove's modification of Dulbecco's medium (IMDM) containing

gentamycin as above, and either NuSerum (Collaborative Research) or fetal bovine serum at 10%.

COS cell transfection for immunofluorescence studies

COS cells at 50% confluence in 6 cm dishes were transfected in a volume of 1.5 ml with a cocktail consisting of DME or IMDM medium containing 10% NuSerum (Collaborative Research), 400 ug/ml DEAE Dextran, 10uM chloroquine diphosphate, and 1 ug/ml DNA. After 4 hours at 37°C (or earlier if the cells appeared ill), the transfection mix was removed and the cells were treated with 10% DMSO in PBS for 2 minutes. Sussman and Milman, Cell Biol. 4:1641-1643 (1984). Cells were then returned to DME/10% calf serum for 48 to 72 hours to allow expression.

Immunoprecipitations, Northern and Southern

T cells were labeled by lactoperoxidase treatment, lysed, and immunoprecipitated by the procedure of Clark and Einfeld (Leukocyte Typing II, Vol. II, pp. 155-167 (1986)), using commercially available goat anti-mouse IgG agarose beads (Cooper Biomedical). COS cells were transfected by DEAE Dextran method and trypsinized and passed without dilution into new plates 24 hours after transfection. 36 hours later, cells were detached by exposure to PBS/EDTA as above, centrifuged, and labeled by the lactoperoxidase method. A cleared lysate was prepared as for the T cell immunoprecipitations, except that the lysis buffer contained 1 mM PMSF, and incubation with the primary antibody was carried out for only 2 hours at 4°C. Eluted samples were fractionated on discontinuous 11.25% polyacrylamide gels using the buffer system of Laemmli (Nature 227:680-685 (1970)).

Northern blot analysis was carried out essentially as described (Maniatis et al., Molecular Cloning, a Laboratory Manual (1982)), except that DMSO was omitted from the loading buffer, denaturation was at 70°C for 5 minutes, and the gel contained 0.6% formaldehyde rather than 6%. The gel was stained in two volumes of water containing 1 ug/ml ethidium bromide, photographed, and transferred to nylon (GeneScreen, DuPont) in

the staining liquor. The transferred RNA was irradiated by exposure to a germicidal lamp through Saran Wrap (Church and Gilbert, Proc. Natl. Acad. Sci. USA 8:1991-1995 (1984)) for 5 minutes at a flux (measured at 254 nm) of 0.22mW/cm². Southern blot analysis was carried out by alkaline transfer to nylon (GeneScreen, DuPont) as described by Reed and Mann (Nucl. Acids Res. 13:7207-7221 (1986)). Hybridization probes were prepared by the method of Hu and Messing (Gene 18:271-277 (1982)), and blots were prehybridized in SDS/phosphate buffer (Church and Gilbert, Proc. Natl. Acad. Sci. USA 8:1991-1995 (1984)) containing 10 DNA microgram equivalents of M13 mp19 phage.

Erythrocyte Rosetting

Erythrocytes were prepared from whole blood by three centrifugations in PBS. COS cells were transfected in 6 cm dishes with CD2 or other surface antigen expression clones by the DEAE method. 48 to 72 hours posttransfection, the medium was aspirated and 2 ml of PBS/5% FDS/azide was added to each plate, followed by 0.4 ml of the appropriate erythrocyte samples as 20% suspensions in PBS. After 1 hour at room temperature, the nonadherent erythrocytes were gently washed off, and the plates examined.

A cDNA encoding CD2 antigen determinants was isolated in the following manner: cDNA was prepared from RNA extracted from the human T Cell tumor line HPB-ALL and inserted into the SV40 origin-based expression vector piH3 as described above. A cDNA library of approximately 3×10^5 recombinants was constructed, and the library was introduced into COS cells by protoplast fusion. Three days later the cells were detached by exposure to EDTA and treated with a pool of monoclonal antibodies, including three (OKT11, Leu5b, and Coulter T11) directed against CD2 determinants. The antibody-treated cells were distributed into dishes coated with an affinity purified sheep anti-mouse IgG antibody, allowed to attach, and separated from the nonadherent cells by gentle washing. This method of enrichment is known in

the immunological literature (Mage et al. J. Immunol. Methods
15:47-56 (1977)).

5 The resulting colonies were pooled, fused into COS cells,
and subjected to a second round of panning as before. In the
third round, a portion of the detached cells was treated with a
mixture of three monoclonal antibodies specific for CD2, and a
Hirt supernatant was again generated and transformed into E.
coli. DNA was prepared from eight of the resulting colonies and
transfected into COS cells. After three days, surface expression
10 of the CD2 antigen was detected by indirect immunofluorescence
in six of eight transfected dishes. Restriction enzyme digestion
of the corresponding plasmid DNAs revealed a 1.5 kb insert in all
six isolates.

One of the six clones was prepared in larger quantities for
further analysis. Following transfection into COS cells,
indirect immunofluorescence analysis with a partial panel of
antibodies provided by the Third International Workshop on
Leukocyte Differentiation Antigens showed that all of the
antibodies provided gave positive reactions with the exception
of one sample which also failed to react with phytohemagglutinin-
activated T lymphocytes. Among the 17 antibodies tested were at
least eight distinguishable groups defined by their differing
patterns of reactivity with lymphocytes of various primate
species. Jonker and Nooij, Leukocyte Typing II, Vol. I, pp. 373-
25 387 (1986).

cdna sequence analysis

30 The CD2 cDNA insert was subcloned into M13 mp19 (Vieira and
Messing, Gene 19:259-268 (1982)) in both orientations, and the
sequence determined by the dideoxynucleotide method (Figure 2).
Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977).
An open reading frame was observed to extend 360 residues from
an ATG triplet satisfying the consensus criteria of Kozak
(Microbiol. Rev.: 1-47:45 (1983)) for translational initiation
codons (Figure 1). The predicted amino acid sequence evokes an

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integral membrane protein with a single membrane spanning hydrophobic anchor terminating in a rather large intracytoplasmic domain. Comparison of the N-terminal amino sequence with the matrix of signal sequence residue frequencies constructed by von Heijne (Nucl. Acids Res. 14:4683-4690 (1986)) suggests that mature CD2 peptide is formed by cleavage of a precursor peptide between the 19th (Ser) and 20th (Lys) residues.

10 A surprising and unexpected feature of this sequence is the presence of a potential N-linked glycosylation site just proximal to the proposed cleavage site. The resulting polypeptide backbone has a predicted molecular weight of 38.9 kd divided into an external domain of mass 21.9 kb and a cytoplasmic domain of mass 14.6 kd. Three N-linked glycosylation sites are present in the extracellular domain.

The membrane spanning domain comprises 26 unchanged residues of predominantly hydrophobic character. In the nine residues immediately following are seven basic residues, either lysines or arginines. The appearance of predominantly hydrophobic residues followed by basic residues is a common organizational feature of transmembrane proteins bearing carboxyl-terminal anchors.

25 Another surprising feature of the transmembrane domain is the appearance of a cys-gly-gly-gly, a beta turn motif (Chou and Fasman, Annual Review of Biochemistry 47:251-276 (1978)), flanked by hydrophobic residues (which are frequently found flanking beta turns). Because only 20 residues arrayed in an alpha helix are theoretically needed to traverse the 3nm membrane bilayer (Tanford, Science 200:1012-1018 (1978)), and as few as 14 hydrophobic residues can allow insertion and export of an integral membrane protein (Adams and Rose, Cell 41:1007-1015 (1985)), the transmembrane segment of the CD2 antigen may contain a bend or kink.

The rather large size of the cytoplasmic domain leaves open the possibility that CD2 possesses an intrinsic enzymatic activity. The cytoplasmic domain is very rich in prolines and contains three sites with high turn probability.

5 Comparison of the amino acid sequence with the NBRF database revealed no substantive homologies with other proteins. In particular, no homology with the T cell receptor alpha or beta chains was observed, ruling out the suggestion that CD2 is a primordial T cell receptor. Milanese et al., Science 231:1118-1122 (1986).

10 Just inside the cytoplasmic face of the protein is a run of basic proteins followed by a serine residue, a pattern found at the same location in both the EGF receptor and the class I histocompatibility genes, and in each case a known site for either in vivo (EGF) and in vitro (HLA) phosphorylation by protein kinase C or cyclic AMP-dependent protein kinase, respectively. Hunter et al., Nature 311:480-482 (1984); Davis and Czech, Proc. Natl. Acad. Sci. 82:1974-1978 (1985); Guild and Strominger, J. Biol. Chem. 259:9235-9240 and 13504-13510 (1984). A similar site is found in the intracytoplasmic domain of the interleukin 2 receptor, and is phosphorylated in vivo by protein kinase C. Leonard et al., Nature 311:626-631 (1984); Nikaido et al., Nature 311:631-635 (1984); Shackelford and Trowbridge, (1984) J. Biol. Chem. 259:11706.

25 Immunoprecipitation of CD2 antigen expressed by transfected cells

COS cells were transfected with the CD2 expression plasmid and surface labeled with ^{125}I by the lactoperoxidase method 60 hours post-transfection. A cell lysate was prepared, and portions were incubated either with monoclonal anti-CD2 antibody (OKT11) or with an extraneous (OKT4; anti-CD4) antibody for 2 hours at 4°C. Sepharose-bound anti-mouse antibody was added, and after several washing steps, the adsorbed proteins were eluted and electrophoresed through a 11.25% acrylamide gel together with similarly prepared immunoprecipitates from phytohemagglutinin-

activated T lymphocytes, the cDNA donor line HPB-ALL, or a long-term T cell line generated in this laboratory. Autoradiography demonstrated a prominent band of immunoreactive material precipitated from transfected COS cells by the anti-CD2 antibody, but not by the control. The calculated mean molecular weight of the COS cell material was 51 kd, compared to a mean molecular weight of 54 kd for the T blast and T cell line material; the antigen from HPB-ALL cells was found to have a molecular weight of approximately 61 kd. The observed differences in size were attributed to different patterns of glycosylation in the different cell types. A minor band of apparent molecular weight 38 kd was present in material immunoprecipitated from COS cells but not from T cells or HPB-ALL cells. The size of this species agrees within experimental error with the predicted molecular weight of mature unglycosylated peptide, 39 kd.

COS cells expressing CD2 form rosettes with sheep erythrocytes

COS cells transfected with the CD2 expression clone were treated for 1 hour with purified MT910 (IgG, kappa) anti-CD2 antibody (Rieber et al., Leukocyte Typing II, Vol. I, pp. 233-242 (1986)) at a concentration of 1 ug/ml, or with purified MB40.5 (IgG1, kappa; Kawata et al., J. Exp. Med. 160:633-651 (1984)) antibody at the same concentration. MB40.5 recognizes a monomorphic HLA-ABC determinant and cross-reacts with African Green Monkey histocompatibility antigens; it was chosen because it represents an isotype-matched antibody recognizing a surface antigen of approximately the same abundance as the CD2 antigen expressed by transfected cells. Sheep erythrocyte rosettes were observed in the presence of MB40.5, but not of MT910. Rosette inhibition was also observed with OKT11 antibody, and not with various other control antibodies.

Transfected COS cells form rosettes with other animal erythrocytes

In addition to sheep erythrocytes, human T cells are known to form rosettes with horse, pig, dog, goat, and rabbit, but not mouse or rat erythrocytes. Johansen et al., J. Allergy Clin. Immunol. 54:86-94 (1974); Amiot et al., in, A. Bernard et al.,

eds., Leucocyte Typing, Springer, publisher, New York, N.Y., pp. 281-293 (1984); Nalet and Fournier, Cell. Immunol. 96:126-136 (1985). Autorosettes between human erythrocytes and human thymocytes (Baxley et al., Clin. Exp. Immunol. 15:385-393 (1973)) have also been reported. COS cells transfected with the CD2 expression clone were treated with either MT910 or with the control antibody, MB40.5, and exposed to erythrocytes from the species above. Rosettes were observed with horse, pig, dog, goat, sheep, rabbit, and human erythrocytes, but not with mouse or rat erythrocytes. Rosette formation was blocked by pretreatment of transfected COS cells with MT910, but not with MB40.5. In these experiments, it was noticed that horse erythrocytes formed unusually dense rosettes, and that goat erythrocytes formed rather sparse rosettes, possibly because their small size made them more susceptible to washing. Mouse erythrocytes showed weak spontaneous binding to the culture dish as well as to MT910 and MB40.5 pretreated cells, while rat erythrocytes showed no detectable binding of any sort.

Binding of human erythrocytes is blocked by LFA3 antibody

Because it has been suggested on the basis of antibody blocking studies that LFA3 is the target structure for the CD2 antigen (Shaw et al., Nature 323:262-264 (1986)), the ability of anti-LFA3 antibody to prevent rosette formation was investigated. Transfected cells were exposed to human erythrocytes pretreated for 2 hours with either anti-LFA3 (IgG1, kappa) as ascites at 1:1000 dilution, or with a 10 ug/ml concentration of each of four isotype-matched nonagglutinating antibodies directed against human erythrocyte antigens as prevalent or more prevalent than LFA3:G10/B11 and D10, anti-K14 antigen, D6, anti-Wr^b antigen; and F7/B9, anti-k antigen. Nichols et al., Vox Sang, in press. The erythrocytes were washed free of excess LFA3 antibody, but were allowed to form rosettes in the presence of the control antibodies to guard against possible loss of antibody blocking power by desorption. Rosette formation was observed in the presence of all four control antibodies, but not with erythrocytes pretreated with anti-LFA3.

COS cells expressing other T cell antigens do not form rosettes

A number of clones were isolated by the same expression technique used to clone CD2 and characterized to varying degrees by antibody reactivity, nucleic acid restriction and sequence analysis, and immunoprecipitation. Representative clones were transfected into COS cells and analyzed for ability to sustain rosette formation. The CD1a, CD1b, CD1c, CD4, CD5, CD6, CD6, CD8, and CD28 (Tp44) clones did not form rosettes with human erythrocytes.

RNA blot analysis

Equal amounts of total RNA prepared from cell types expressing or lacking CD2 antigen were electrophoresed through denaturing agarose gels and transferred to nylon. Hybridization of the transferred RNA with a strand selective probe (Hu and Messing, Gene 17:271-277 (1982)) prepared from an M13 clone containing a CD2 cDNA insert revealed the presence of prominent 1.65 and 1.3 kb transcripts present in RNA derived from thymocyte, activated T cell, and senescent T cell populations. Lesser amounts were found in RNA extracted from the cDNA donor line, HPB-ALL and less still from MOLT4; barely detectable levels were recorded in RNA from the HSB-2 line. No reactivity was observed with RNA from Namalwa (Burkitt lymphoma), U937 (histiocytic leukemia), HuT-78 (Adult T cell leukemia), PEER (T cell leukemia), or Jurkat clone J3R7 (T cell leukemia) lines. The pattern of reactivity conformed well with the known or measured pattern of expression of CD2 antigen, which was absent or undetectable on the Namalwa, *937, HuT-78, J3R7, PEER, and HSB-2 cell lines, weakly present on MOLT4, more strongly present on EPB-ALL, and most strongly present on activated T cells. Thymocytes are also known to express high levels of CD2 antigen.

Examination of the sequence of the cDNA clone suggested that the 1.3 kb RNA might arise by formation of an alternate 3' end distal to the canonical polyadenylation signal AATAAA at position 1085 in the cDNA sequence. To test this notion, RNA from HPB-ALL and activated T cells was subjected to Northern blot analysis and

hybridized either with a complete cDNA probe, or with a probe derived from the 3' portion of the cDNA distal to nucleotide 1131. The latter probe reacted only with the 1.65 kb species, while the former showed the same reactivity pattern observed in Figure 5. This result is consistent with the suggested origin of the 1.3 kb transcript.

In both activated and senescent T cell RNA preparations, a weakly hybridizing transcript of approximately 0.75 kb was detected. At present the origin of this RNA is unknown.

Genomic organization of the CD2 gene

Southern blot analysis of genomic DNA from placenta, peripheral blood lymphocytes, T cells, HeLa cells, or the tumor lines used in the RNA analysis above showed identical BamHI digest patterns, indicating that rearrangement is not involved in the normal expression of the CD2 gene during development. Similarly, no gross genomic alteration underlies the failure of the examined T cell tumor lines to express CD2 antigen. Restriction analysis of total genomic DNA with a number of other enzymes, as well as preliminary results with an incomplete collection of 1 phage recombinants bearing the CD2 sequence, shows that the gene is divided into at least four exons.

Example II Isolation and Molecular Cloning of Human LFA-3 Antigen

The previous example shows that cDNAs encoding surface antigens, such as the CD2 antigen, can be isolated by the transient expression system of the present invention, in which COS cells transfected with cDNA libraries are allowed to attach to ("panned" on) antibody-coated plates. Plasmid DNA is recovered from cells adhering to the plates, transformed into *E. coli*, and the process is repeated, usually twice, to isolate the desired clone. Although powerful, this approach cannot be used when the monoclonal antibodies used for panning recognize determinants on the untransfected cells. This appears to be the case for anti-LFA3 monoclonal TS2/9. However, a similar transient expression system based on polyoma virus replication-

competent cells should allow almost all monoclonals to be used, since the probability of cross reaction between murine antibodies and murine cell surface determinants should usually be small.

5 A new expression vector, CDM8 (Figure 3) was created from the COS cell vector piH3M described previously. The new vector differs by the inclusion of a deleted version of a mutant polyoma virus early region selected for high efficiency expression in both murine and monkey cells, by the replacement of substantially all of the human immunodeficiency promoter region with the cognate sequences of the human cytomegalovirus immediate early promoter, and by inclusion of a bacteriophage T7 promoter between the eukaryotic promoter and the site of cDNA insertion. Expression in COS cells of chloramphenicol acetyltransferase by all of the vectors was equivalent.

10 A library of 1.9×10^6 recombinants having inserts greater than 0.8 kb in size was prepared in the CDM8 vector from a microgram of poly A+ RNA isolated from the human lymphoblastoid cell line JY. The library was introduced into WOP cells (NIH 3T3 cells transfected with polyoma origin deletion DNA) by spheroplast fusion, and subjected to three rounds of panning and reintroduction into E. coli as described in Example I.

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25 200
A clone encoding the LFA-3 antigen was identified by indirect immunofluorescence of transfected WOP cells, amplified and sequenced (Figure 4). Within the 874 bp insert, an open reading frame of 237 residues originates at a methionine codon closely corresponding to the consensus sequence suggested by Kozak, Microbiol. Rev. 47:1-45 (1983). The reading frame terminates in a series of hydrophobic residues lacking the characteristic basic anchoring residues of internal membrane proteins, but sharing features with known phosphatidylinositol-linked superficial membrane proteins. The features include clustered serine or threonine residues in a hydrophilic region immediately preceding the hydrophobic domain, and the presence of serines and threonines in the hydrophobic portion.

5 The amino acid sequence predicted from the nucleotide
sequence of the LFA-3 clone was compared to the NBRF database,
and no significant homologies were uncovered; the most
significant scores were to the HIV envelope protein. Within the
200 residues comprising the presumed mature protein are 6 N-
linked glycosylation sites, and 5 tandem serine or tandem
threonine residues that frequently appear in O-linked
glycosylated proteins. Ten cysteine residues appear in the
complete sequence, 6 of which are distributed in the latter half
10 of the mature protein, and one of which falls in the carboxy-
terminal hydrophobic domain. Although esterification of cysteine
thiols to fatty acids is a common occurrence in integral membrane
proteins, and may play an alternate role in membrane anchoring
of LFA-3, two examples are known of cysteine residues within or
at the margin of the hydrophobic region of phosphatidylinositol
linked proteins.

25 The predicted sequence suggests that the known manipulations
for increasing erythrocyte adhesion to T cells may find direct
physical explanation in the structure of the LFA-3 molecule.
Aminoethylisothiuronium bromide, the thiourea adduct of
bromoethylamine, undergoes spontaneous rearrangement to
mercaptoethylguanidine at alkaline pH. The latter likely gains
access to disulfide bonds inaccessible to less chaotropic
reducing agents and may thereby reduce and promote the unfolding
of the LFA-3 molecule. Similarly, neuraminidase may decrease
steric interference by the many carbohydrate chains on the
molecule.

30 RNA and DNA blot hybridization analysis showed that the LFA-
3 gene shares no closely related sequences in the genome, and
encodes a single RNA species of about 1 kb in length. Cell lines
that express large amounts of surface LFA-3 have greater amounts
of LFA-3 RNA than those that express small or nondetectable
amounts.

Radioimmunoprecipitation of the antigen expressed in transfected COS and murine cells shows a broad band of approximately 50 kd mean molecular mass, similar to that found in JY cells.

5 Example III Isolation and Molecular Cloning of the Human CD28 cDNA Antigen

10 The previous examples illustrate the monoclonal antibody-based technique of the present invention for enrichment of cDNAs encoding surface antigens. In the present example, a method of constructing plasmid expression libraries is described which allows the enrichment technique to be fully exploited. The method of the present invention for making plasmid expression libraries is of general use for expression cloning.

The antibody selection technique of the present invention has also been applied to isolate a cDNA clone encoding the CD28 antigen. The antigen shares substantial homology with members of the immunoglobulin superfamily and forms a dimer structure on the surface of transfected COS cells similar to the dimer structure found on T lymphocytes.

Preparation of cDNA Libraries

25 Poly(A)+ RNA was prepared from the human T-cell tumor line HPB-ALL by oligo(dT) cellulose chromatography of total RNA isolated by the guanidinium thiocyanate method (Chirgwin, J.M. et al., Biochemistry 18:5294-5299 (1979)). cDNA was prepared by a protocol based on the method of Gubler and Hoffman (Gubler, U. et al., Gene 25:263-269 (1982)). 4 ug of mRNA was heated to approximately 100°C in a 1.5 ml centrifuge tube for 30 seconds, quenched on ice, and the volume adjusted to 70 ul with RNase-free water. To this were added 20 ul of buffer (0.25 M Tris pH 8.8 (8.2 at 42°C), 0.25 M KCl, 30 mM MgCl₂), 2ul of RNase inhibitor (Boehringer 36 u/ul), 1 ul of 1M DTT, 1 ul of 5 ug/ul of oligo dT (Collaborative Research), 2 ul of 25 mM each deoxynucleoside triphosphate (US Biochemicals), and 4 ul of reverse transcriptase (Life Sciences, 24 u/ul). After 40 minutes at 42°C, the reaction was terminated by heating to 70°C for 10 minutes. To the

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reaction mix was then added 320 ul of RNase free water, 80 ul of buffer (0.1 M Tris pH 7.5, 25 mM MgCl₂, 0.5 M KCl, 0.25 mg/ml BSA, and 50 mM DTT), 25 units of DNA Polymerase I (Boehringer), and 4 units of RNase H (BRL). After 1 hour at 15°C and 1 hour at 22°C, 20 ul of 0.5M EDTA pH 8.0 were added, the reaction mixture was extracted with phenol, NaCl was added to 0.5 M, linear polyacrylamide (carrier; Strauss, F. et al., Cell 37:889-901 (1984)) was added to 20 ug/ml, and the tube was filled with ethanol. After centrifugation for 2-3 minutes at 12,000 x g, the tube was removed, vortexed to dislodge precipitate spread on the wall of the tube, and respun for 1 minute.

Unpurified oligonucleotides having the sequence CTCTAAAG and CTTTAGAGCACA were dissolved at a concentration of 1 mg/ml, MgSO₄ was added to 10 mM, and the DNA precipitated by adding 5 volumes of EtOH. The pellet was rinsed with 70% ETOH and resuspended in TE at a concentration of 1 mg/ml. 25 ul of the resuspended oligonucleotides were phosphorylated by the addition of 3 ul of buffer (0.5 M Tris pH 7.5, 10 mM ATP, 20 mM DTT, mM spermidine, 1 mg/ml BSA, and 10 mM MgCl₂) and 20 units of polynucleotide kinase followed by incubation at 37°C overnight.

3 ul of the 12-mer and 2 ul of the 8-mer phosphorylated oligonucleotides were added to the cDNA prepared as above in a 300 ul reaction mixture containing 6 mM Tris pH 7.5, 6 mM MgCl₂, 5 mM NaCl, 0.35 mg/ml BSA, 7 mM mercaptoethanol, 0.1 mM ATP, 2 mM DTT, 1 mM spermidine and 400 units T4 DNA ligase (New England BioLabs) at 15° overnight. 10 ul of 0.5 M EDTA were added, the reaction was phenol extracted, ethanol precipitated, resuspended in a volume of 100 ul and layered on a 5 ml gradient of 5-20% potassium acetate in 1 mM EDTA, 1 ug/ml ethidium bromide. The gradient was spun 3 hours at 50,000 rpm (SW55 rotor) and fractionated manually, collecting three approximately 0.5 ml fractions followed by six approximately 0.25 ml fractions in microcentrifuge tubes by means of a butterfly infusion set inserted just above the curve of the tube. Linear polyacrylamide was added to 20 ug/ml, the tubes were filled with ethanol,

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5 chilled, spun, vortexed and respun as above. The precipitate was washed with 70% ethanol, dried, and resuspended in 10 ul. 1 ul of the last 6 fractions was run on a gel to determine which fractions to pool, and material less than 1 kb in size was typically discarded. Remaining fractions were pooled and ligated to the vector.

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10 The complete sequence and derivation of the vector is shown in Figure 5. The vector was prepared for cloning by digestion with BstXI and fractionation on 5-20% potassium acetate gradients as described for the cDNA. The appropriate band was collected by syringe under 300 nm UV light and ethanol precipitated as above. cDNA and vector were titrated in test ligations. Usually 1-2 ug of purified vector were used for the cDNA from 4 ug of poly A+ RNA. The ligation reactions were composed as described for the adaptor addition above. The ligation reactions were transformed into MC1061/p3 cells made competent as described above. The transformation efficiency for supercoiled vector was $3-5 \times 10^8$ colonies/ug.

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20 Recovery and characterization of the CD28 clone

25 Panning of the library was carried out as described herein above, using purified antibody 9.3 (DuPont) at a concentration of 1 ug/ml in the antibody cocktail. The methods used for COS cell transfection, radioimmunoprecipitation, RNA and DNA blot hybridization, and DNA sequencing were all as described herein above.

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30 To isolate the CD28 cDNA, a large plasmid cDNA library was constructed in a high efficiency expression vector containing an SV40 origin of replication. A preferred version of the vector, containing an M13 origin, is shown in Figure 6. Three features of the vector make it particularly suitable for this use: (i) the eukaryotic transcription unit allows high level expression in COS cells of coding sequences placed under its control; (ii) The small size and particular arrangement of sequences in the plasmid permit high level replication in COS cells; and (iii) the

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presence of two identical BstXI sites in inverted orientation and separated by a short replaceable fragment allows the use of an efficient oligonucleotide-based strategy to promote cDNA insertion in the vector.

5 The BstXI cleavage site, CCAN'₅NTGG, creates a four base 3' extension which varies from site to site. A vector was created in which two identical sites were placed in inverted orientation with respect to each other, and separated by a short replaceable segment of DNA. Digestion with BstXI followed by removal of the
10 replaceable segment yielded a vector molecule capable of ligating to fragments having the same ends as the replaceable segment, but not to itself. In parallel, cDNA synthetic oligonucleotides were employed that give the same termini as the replaceable segment. The cDNA then could not ligate to itself, but could ligate to the vector. In this way, both cDNA and vector were used as efficiently as possible.

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25 Tailing with terminal transferase achieves the same end, but with less convenience and less overall efficiency. Moreover, homopolymer tracts located 5' to cDNA inserts have been reported to inhibit expression in vitro and in vivo (Yokota, T., et al., Nucl. Acids Res. 14:1511-1524 (1986); Riedel, H., EMBO J. 3:1477-1483 (1985)). Similar approaches based on the use of partially filled restriction sites to favor insertion of genomic DNAs (Zabarovsky, E.R., et al., Gene 42:119-123 (1986)) and cDNAs (Yang, Y., et al., Cell 47:3-10 (1986)) recently have been reported. These approaches give 2 or 3 base complementary termini, which usually ligate less efficiently than the 4 base extensions reported here.

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Although the cloning scheme of the present invention does not result in a directional insertion of the cDNA, the ability to make large libraries easily, coupled with a powerful selection procedure, makes directional insertion unnecessary. The library construction efficiencies observed according to the present invention, between 0.5 and 2×10^6 recombinants per ug of mRNA,

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with less than 1% background and an insert size greater than 1 kb, compared favorably with those described for phage vectors lambda gt10 (7.5×10^5 /ug of mRNA) and lambda gt11 (1.5×10^6 /ug of mRNA) (Huynh, T., et al., In: DNA Cloning Vol. I, A Practical Approach, Glover, D.M. (ed.), IRL Press, Oxford (1985), pp. 49-78); but the resulting clones were more convenient to manipulate.

Surface antigen cDNAs can be isolated from these libraries using the antibody enrichment method of the present invention. In this method, the library is introduced into COS cells (for example, by spheroplast or protoplast fusion), where it replicates and expresses its inserts. The cells are harvested by detaching without trypsin, treated with monoclonal antibodies specific for the surface antigens desired, and distributed in dishes coated with affinity purified antibody to mouse immunoglobulins. Cells expressing surface antigen adhere, and the remaining cells can be washed away. From the adherent cells, a Hirt fraction is prepared (Hirt, B., J. Molec. Biol. 26:365-369 (1967)), and the resulting DNA transformed back into E. coli for further rounds of fusion and selection. Typically, after two rounds of selection with monoclonal antibodies recognizing different surface antigens, a single round of selection is performed with a single antibody, or pool of antibodies recognizing the same antigen.

Isolation of a CD28 cDNA

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The CD28 cDNA was isolated from a library of about 3×10^5 recombinants prepared from cDNA from 0.8 ug of poly A⁺ RNA using an earlier version of the protocol described in the Materials and Methods. The library was screened for CD28 (and other surface antigen) cDNA clones by the method outlined above. After the third transfection, COS cells were panned with the 9.3 antibody alone. A Hirt supernatant was prepared from the adherent cells and transformed into E. coli. Plasmid DNA was isolated from eight colonies and transfected individually into COS cell cultures. The presence of the CD28 antigen was detected in three

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of eight transfected cultures by indirect immunofluorescence. All three plasmid DNAs contained an insert of about 1.5 kb.

cDNA sequence analysis

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10 The CD28 cDNA encodes a long open reading frame of 220 residues having the typical features of an integral membrane protein (Figure 7). Removal of a predicted (von Heijne, Nucl. Acids Res. 14:4683-4690 (1986)) N-terminal signal sequence gives a mature protein of 202 residues comprising an extracellular domain with five potential N-linked glycosylation sites (Asn-X-Ser/Thr), a 27-amino acid hydrophobic membrane spanning domain, and a 41-amino acid cytoplasmic domain. Comparison of the amino acid sequence of CD28 with the National Biomedical Research Foundation database (Version 10.0) revealed substantial homology with mouse and rabbit immunoglobulin heavy-chain variable regions over a domain spanning almost the entire extracellular portion of CD28. Within this domain two cysteine residues in the homology blocks Leu-(Ser or Thr)-Cys and Tyr-(Tyr or Phe)-Cys are shared by CD28, CD4, CD8, immunoglobulin heavy- and light-chain variable sequences and related molecules with approximately the same spacing (Maddon et al., Annu. Rev. Biochem. 48:961-997 (1979)).

CD28 cDNA directs the production of a homodimer in transfected COS cells

25 Immunoprecipitation of CD28 antigen from transfected COS cells was carried out using the monoclonal antibody 9.3 (Hansen, J.A., et al., Immunogenetics 10:247-260 (1980)). The material obtained from COS cells migrated with a molecular weight of 74 kd under nonreducing conditions and 39 kd under reducing conditions, a pattern consistent with homodimer formation. Under
30 the same conditions activated T cells give bands with molecular weights of 87 and 44 kd, and HPB-ALL cells give bands of 92 and 50 kd, under nonreducing and reducing conditions respectively. The variation in molecular weight of the material obtained from different cell types arises as a result of differing
35 glycosylation patterns characteristic of each type. Similar results were observed with other leukocyte surface antigens (Seed

et al., Proc. Natl. Acad. Sci USA 87 (1987)). The nucleotide sequence of the CD28 cDNA predicts a mature protein with molecule weight of 23 kd, much smaller than observed in these experiments, and probably attributable to utilization of the 5 N-linked glycosylation sites predicted by the amino acid sequence.

RNA blot analysis

Equal amounts of total RNA prepared from cell types expressing or lacking CD28 were subjected to RNA blot analysis as described hereinabove. Four bands with molecular weights of 3.7, 3.5, 1.5, and 1.3 kb were visible in lanes containing RNA from thymocytes, T blasts, senescent T cells, and the T cell leukemia cell lines PEER and HPB-ALL. No bands were detected in lanes containing RNA prepared from the cell lines U937 (histiocytic leukemia), HuT-78 (Adult T cell leukemia), Jurkat (T cell leukemia), Namalwa (Burkitt lymphoma), MOLT4, and HSB-2, all of which do not express CD28. The 1.5 kb transcript presumably corresponds to the isolated cDNA, and the 3.7 and 3.5 kb species reflect incomplete splicing or alternative polyadenylation site utilization. The 1.3 kb transcript may terminate at an unconventional polyadenylation signal, since there is no obvious candidate in the sequence.

The CD28 gene is not rearranged

DNA blot analysis (Seed et al., Proc. Natl. Acad. Sci USA 87 (1987)) of genomic DNA from placenta, peripheral blood lymphocytes, T cells, HeLa cells, or the tumor lines used in the RNA blot analysis above showed identical Dra 1 digest patterns indicating that rearrangement is not involved in the normal expression of the CD28 gene during development. Similarly, no gross genomic rearrangement underlies the failure of the examined T-cell tumor lines to express CD28 antigen. It may be inferred from the Dra 1 fragment pattern that the CD28 gene contains at least two introns.

Example IV Isolation and Molecular Cloning of Two Human CD7 Antigen cDNAs

5 The CD7 cluster of antibodies (Palker, et al., Leukocyte Typing II, Springer-verlag, New York, 303-313 (1985)) recognized a 40 kd glycoprotein (gp40) on the surface of peripheral blood T cells and thymocytes. Early studies with anti-CD7 antibodies showed that CD7⁺ T cells enhance immunoglobulin (Ig) synthesis by B cells (Miroshima et al., J. Immunol. 129:1091-1098 (1982)), suppress B cell Ig synthesis when stimulated with Concanavalin A (Haynes et al., Proc. Natl. Acad. Sci. U.S.A. 76:5829-5833 (1979)) and are the precursors of the cytotoxic T cells generated in mixed lymphocytic culture (Morishima et al., J. Immunol. 129:1091-1098 (1982)). Furthermore, CD7 has been found to be the most reliable marker for the identification of T cell acute lymphoblastic leukemia (Link et al., Blood 62:722-728 (1983)). As such, studies have been carried out, in which cytotoxins coupled to the anti-CD7 antibody 3A1 were used to purge bone marrow prior to reinfusion to avoid early relapse in autologous bone marrow transplants or as prophylaxis against graft vs. host disease in allogenic bone marrow transplants (Ramakrishnan et al., J. Immunol. 135:3616-3622 (1985)). Similarly, anti-CD7 antibodies also show promise as immunosuppressive agents in the treatment of allograft rejections (Raftery et al., Transpl. Proc. 17:2737-2739 (1985)) which is in accord with the recent observation that the anti-CD7 antibody 7G5 significantly inhibits the primary mixed lymphocyte reaction (Lazarovits et al., Leukocyte Typing III. Oxford Univ. Press, Oxford (1987)).

At present the physiological role of CD7 is not understood. It is known that anti-CD7 antibodies are not mitogenic, and do not block the T cells' response to PHA, or tetanus toxoid (Palker et al., Leukocyte Typing, Springer-Verlag, New York, 303-313 (1985)). Some have noted that expression of CD7 in thymocytes occurs prior to the onset of T cell receptor beta-chain rearrangement (Pittaluga et al., Blood 68:134-139 (1986)) and have pointed to a possible role for CD7 in this rearrangement and subsequent expression of the T cell receptor. It is clear that the cloning of the CD7 antigen would further efforts to

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5 Bovine erythrocytes for rosette assays were prepared as described by Ercolani et al., J. Immunol. 127:2044-2051 (1981). Briefly, a 2% suspension of bovine erythrocytes was washed with PBS/0.5% BSA and treated with subagglutinating amounts of either antibodies at 4°C for 1 hour. Erythrocytes were then washed twice with PBS/0.5% BSA and adjusted to a 2% solution. 2 ml of antibody-coated erythrocytes were layered on 60 mm dishes containing COS cells which had been transfected 48 hours earlier with either CD7, CD32 or CD28 by the DEAE Dextran method. The dishes were then centrifuged at 150 X g at 4°C for 15 minutes. After an additional 45 minute incubation at 4°C, the plates were gently washed 5 times with 5 mls of PBS/0.5% BSA, and the COS cells were examined for rosette formation. These experiments were also performed in the presence of 0.1% sodium azide without alteration of the results.

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25 Formation of T cell rosettes with antibody-coated erythrocytes

Peripheral blood lymphocytes were obtained from heparinized blood by centrifugation at 4°C over a Ficoll-Hypaque gradient at 400 x g for 30 minutes. Leukocytes at the interface were washed two times with PBS. The leukocytes were adjusted to 10Y7 cells/ml in IMDM/10% Fetal Bovine Serum (FBS) and incubated in tissue culture dishes at 37°C for 30 minutes. Nonadherent cells were transferred to new dishes, and PHA was added to stimulate proliferation of T lymphocytes. On the next day the cells were washed with PBS and placed in fresh IMDM/10%FBS.

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Rosette assays were performed three days later. Cells were washed with PBS/0.5% BSA, and a 10 ul suspension of 2% Ig-coated erythrocytes prepared as described above was added to 10 ul of PBS/0.5% BSA containing 5×10^6 cells/ml. The mixtures were placed in Falcon round bottom 96 well plates and centrifuged at 150 X g for 15 min at 4°C. After an additional incubation of 45 min at 4°C pellets were resuspended with 10 ul of PBS/0.5% BSA, and the rosettes scored by phase contrast microscopy. The

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experiments were carried out in both the presence and absence of 0.1% sodium azide with no detectable difference.

Isolation of cDNAs encoding the human CD7 antigen

To isolate CD7 cDNAs, a large plasmid library was constructed in the expression vector π 3M as describe hereinabove. The library was introduced into COS cells by spheroplast fusion, and allowed to replicate and express its inserts. The COS cells were harvested by detaching without trypsin 48 to 72 hours after transfection, treated with monoclonal antibodies specific for surface antigens believed to be encoded in the library, and distributed in dishes coated with affinity purified anti-mouse antibody as described herein. Under these conditions, cells expressing surface antigen adhere and the remaining cells can be washed away.

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A Hirt (Hirt, J. Mol. Biol. 26:365-369 (1967)) fraction was prepared from adherent cells, and the resulting DNA transformed back into E. coli for further rounds of fusion and selection. In the third round of selection the detached cells were treated with a mixture of monoclonal antibodies specific for CD7 (765 and Leu9), and a Hirt supernatant was again generated and transformed into E. coli. After transformation of the DNA into E. coli 8 colonies were picked, and the plasmid DNA prepared from them by an alkaline miniprep procedure (Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982)). DNA was prepared from 8 resulting colonies and transfected into COS cells. After 3 days, surface expression of the CD7 antigen was detected by indirect immunofluorescence in 7 of 8 transfected dishes. Restriction enzyme digest of the corresponding plasmid DNAs revealed two species. One contained a 1.2 kb insert, and the other a 1.3 kb insert.

CD7 cDNA sequence analysis

Both isolates were sequenced by the dideoxynucleotide method. The 1.2 kb cDNA encodes a long open reading frame of 240

residues having the typical features of an integral membrane protein. The initial assignment of the signal sequence cleavage site by the method of von Heijne (Nucl. Acids Res. 14:4683-4690 (1986)) was at the 18th residue. It later was determined, however, that the homology with immunoglobulin variable regions would better predict the mature terminus at residue 26; this assignment would also correlate well with the position of the intron as discussed below and as shown in Figure 8. Removal of the predicted N-terminal signal sequence gives a mature protein of 215 residues with a predicted molecular mass of 23 kd. In the extracellular domain are two N-linked glycosylation sites (Asn-X-Ser Thr), in agreement with the results of Sutherland et al. (J. Immunol. 133:327-333 (1984)), who also showed the presence of O-linked glycans and covalently associated palmitic acid on the mature protein. In the 27 amino acid hydrophobic membrane spanning domain is a single cysteine residue which may be the site of fatty acylation (Rose et al., Proc. Natl. Acad. Sci. USA 81:2050-2054 (1984); Kaufman et al., J. Biol. Chem. 259:7230-7238 (1984)). The length of the cytoplasmic domain, 39 residues, is in good agreement with the 30-40 amino acids predicted by protease digestion of the CD7 precursor in rough microsomal membrane fractions (Sutherland et al., J. Immunol. 133:327-333 (1984)).

Sequence analysis of the 1.7 kb clone (Figure 8) revealed the presence of an intron located 121 bp from the 5' end. The 411 bp intron contains stop codons in all three reading frames and is located just downstream of the secretory signal sequence, as is frequently observed for secreted or surface proteins. Both the 5' and 3' ends of the intron conform to the splice donor/acceptor consensus AAG GTRAGA/.../Y₆₋₁₁NYAG A (Mount, Nucl. Acids Res. 10:459-472 (1982)). Because both the 1.2 and 1.7 kb clones express CD7 antigen equally well in COS cells, the intron must be excised in COS cells fairly efficiently.

Comparison of the amino acid sequence with the National Biomedical Research Foundation database revealed substantial

homology with human and mouse immunoglobulin kappa chain and T-cell receptor gamma chain variable regions over almost the entire extracellular portion of the molecule. Two cysteine residues shared in approximately equal spacing by all three structures fall in the conserved sequences Ile-Thr-Cys and Tyr-X-Cys. In kappa chain variable regions these cysteines form a disulfide bridge. The presence of at least one intrastrand disulfide bond in the CD7 structure has previously been proposed by Sutherland et al. (J. Immunol. 133:327-333 (1984)), who noted that immunoprecipitation of CD7 gave rise to a band with an apparent molecular mass of 40 kd under reducing conditions and 38 kd under nonreducing conditions.

Based on the homology with immunoglobulin V-regions, it is predicted that CD7 contains a disulfide bond linking Cys 23 and Cys 89. A second disulfide bond, linking Cys 10 and Cys 117, has been proposed, based on the structural similarity between CD7 and Thy-1. The extracellular domains of both Thy-1 and CD7 have 4 cysteine residues, in roughly homologous positions. The 4 cysteine residues of Thy-1 are joined in two internal disulfide bridges between Cys 9-111 and Cys 19-85 (Williams et al., Science 216:696-703 (1982)). In Thy-1, Cys 111 forms an amide bond with the ethanolamine moiety of a substituted phosphatidylinositol, and is thus the last residue of the mature molecule (Tse et al., Science 230:1003-1008 (1985)). In CD7, Cys 117 is followed by four repeats of a sequence whose consensus is Xaa-Pro-Pro-Xaa-Ala-Ser-Ala-Leu-Pro, and which, it is proposed, plays the role of a stalk projecting the V-like domain away from the surface of the cell.

In addition to the homologies shown in Figure 20 and mentioned above, the extracellular domain of CD7 has significant homology with both chains of the rat CD8 heterodimer (Johnson et al., Nature 323:74-76 (1986)), and the myelin P₀ protein (Lemke et al., Cell 40:501-508 (1985)).

CD7 directs the production of a 40 kd protein in transfected COS cells

Immunoprecipitation of CD7 antigen from transfected COS cells was carried out as described herein using monoclonal antibody 7G5 (Lazarovits et al., Leukocyte Typing III, Oxford Univ. Press, publisher, Oxford, England (1987). The material obtained from COS cells migrated with as a broad band with molecular weight of 40 kd under reducing conditions. Under the same conditions HPB-ALL cells (the cDNA donor line) and activated T cells gave bands with molecular widths of 41 and 39 kd respectively. In both the COS cell and HPB-ALL lane a faint band with molecular weight of 30 kd was also observed, possibly corresponding to a partially glycosylated precursor (Sutherland, D.R., et al., J. Immunol. 133:327-333 (1984)).

RNA blot analysis

Equal amounts of total RNA prepared from cell types expressing or lacking CD7 were subjected to Northern blot analysis as described herein. A single 1.3 kb species was visible in lanes containing RNA from thymocytes, activated T cells, resting T cells, and the T cell leukemia lines HuT-78, HPB-ALL, Jurkat J3R7, HSB-2 and PEER. With the exception of the PEER cell line, none of the T cell tumors showed significant overexpression of CD7 transcripts. CD7 RNA was detected in all of the thymus-derived cells, but not in RNA from U937 (histiocytic leukemia) and Namalwa (Burkitt Lymphoma) cells. No band corresponding to the 1.7 kb cDNA could be detected, suggesting that this species is artificially enriched during the cloning or library amplification process.

Enrichment during amplification seems unlikely because the 12 kb cDNA clone propagates as well in E. coli as the 1.7 kb clone. However, immediately upstream and downstream from the site of insertion of the intron are sequences that could form an interrupted stem and loop structure. Eight of the 10 basepairs of the potential stem are GC pairs, perhaps giving the structure sufficient stability to interfere with elongation of the cDNA first strand. The presence of the intron greatly separates the two halves of the stem, potentially eliminating the structure via

unfavorable loop entropy and allowing efficient first strand synthesis.

The CD7 gene is not rearranged

Southern blot analysis of genomic DNA from placenta, peripheral blood lymphocytes, T cells, HeLa cells, or the tumor lines used in the RNA blot analysis above showed identical Dra 1 digest patterns. Thus, the CD7 gene is not grossly altered during development, and the high level of expression in the PEER cell line is not the consequence of a substantial genomic rearrangement.

COS cells expressing CD7 do not bind IgM

Human peripheral blood T lymphocytes express receptors for IgM antibodies (FcRu: Moretta et al., Eur. J. Immunol. 5:565-569 (1975); McConnell et al., Immunol. 30:835-837 (1976)). Recently it has been reported that CD7 might play a role in IgM binding by T cells (Sandrin et al., Leukocyte Typing III, Oxford Univ. Press, publisher, Oxford, England (1987)). L cells, normally CD7⁻ and FcRu⁻, become CD7⁺ and FcRu⁺ when transfected with a 16 kb genomic fragment encoding the CD7 antigen (Sandrin et al., Leukocyte Typing III, Oxford Univ. Press, publisher, Oxford, England (1987)). Furthermore, IgM binding to CD7-positive cells can be blocked by the anti-CD7 monoclonal antibody Huly-m2 (Thurlow et al., Transplantation 38:143-147 (1984)), and IgM columns bind a 37 kd protein from radiolabeled lysates of peripheral blood T lymphocytes (Sandrin et al., Leukocyte Typing III, Oxford Univ. Press, publisher, Oxford, England (1987)).

Accordingly, COS cells expressing CD7 were tested for their ability to bind IgM. IgM receptor activity was assayed either by direct binding (Hardin et al., Proc. Natl. Acad. Sci. USA 76:912-914 (1979)) or by a rosette assay with ox erythrocytes coated with an IgM fraction of rabbit anti-bovine red cell serum as described by Ercolani et al., J. Immunol. 127:2044-2051 (1981)). Cells expressing CD7 neither bound human IgM nor formed rosettes with IgM-coated erythrocytes. Under the same

conditions, COS cells transfected with a cDNA encoding the human IgG receptor CDw32 bound IgG directly and formed rosettes with IgG-coated erythrocytes. Erythrocytes coated with IgM or IgG antibodies also adhered to a fraction of peripheral blood lymphocytes as reported (Moretta et al., Eur. J. Immunol. 5:565-569 (1975)).

These results do not support the notion that the CD7 antigen is by itself an IgM receptor, although they do not exclude the possibility that COS cells suppress IgM binding activity in some manner, or that CD7 is part of, or modified to become, an IgM receptor. That CD7 is not by itself an IgM receptor is supported by the observation that a number of CD7⁺ T cell lines are FcR γ (Sandrin et al., Leukocyte Typing III, Oxford Univ. Press, publisher, Oxford, England (1987)).

Example V Isolation and Molecular Cloning of the Human CDw32 Antigen

A cDNA encoding the human CDw32 antigen, a human receptor for immunoglobulin G constant domains (Fc receptor), was isolated by the method of the present invention, by virtue of its affinity for its ligand, IgG. The sequence of the isolated clone is most closely related to the murine beta 2 Fc receptor, but has diverged completely in the portion encoding the cytoplasmic domain. The receptor expressed in COS cells shows a preference for IgG₁ among IgG subtypes, and no affinity for IgM, IgA or IgE.

To isolate the Fc receptor clone, cDNA libraries were prepared from tumor cell lines or from a human tumor and transfected into COS cells. After 48 hours, the cells were treated with mouse or human IgG antibodies, and allowed to settle on dishes coated with affinity-purified sheep anti-mouse IgG or goat anti-human IgG antibodies. After lysis, DNA recovery, and transformation in E. coli, the cycle was repeated for two more rounds. Although no positive clones were isolated from the tumor line libraries, a cDNA clone encoding an Fc receptor was isolated from a library prepared from a human adrenal tumor. It has been discovered that many tumors are heavily infiltrated by

macrophages and lymphocytes. Thus, tumor RNA may be a productive source in general for transcripts of human macrophages.

By indirect immunofluorescence assay, the human receptor expressed on COS cells bound all mouse and human IgGs with relatively low affinity ($10^{-7}M$), and a clear discrimination was noted among human antibodies for IgG₁. Human IgM, IgA₁, IgA₂, and IgE did not bind, nor did murine IgM or IgA. As expected, human Fc, but not Fab fragments, bound to the transfected cells. Among monoclonal antibodies donated to the Third International Workshop on Leukocyte Differentiation Antigens, three gave strong positive immunofluorescence: two (out of two) recognizing the Fc Receptor CDw32 determinant, and one (out of four) recognizing the CD23 (B cell IgE Fc receptor) determinant. Monoclonals recognizing the T cell/Macrophage Fc receptor antigen CD16 gave only weak immunofluorescence comparable to that shown by control ascites.

Radioimmunoprecipitation of transfected COS cells with CDw32 antibodies showed the presence of a single 40 kd species, comparable in size to the antigen recognized on the surface of the myeloid CDw32⁺ line HL-60, and to the less abundant antigen present on the histiocytic leukemia line U937. This result reinforces the notion that the isolated receptor is CDw32, as the CD16 receptor is reported to be substantially larger (60-70 kd).

The nucleotide sequence of the isolated receptor (Figure 9) is highly homologous to that of members of the recently isolated murine receptor family, and most closely related to the murine beta₂ receptor by nucleic acid homology. Surprisingly, the murine beta₂ receptor is found on T and B lymphocytes and macrophages, while the alpha receptor is restricted to macrophages; in the human system, CDw32 (shown here to be beta₂-like) is restricted to macrophages while another Fc receptor (CD16) is found on lymphocytes and macrophages. The human sequence appears to have diverged from the mouse sequence by insertion of approximately 1 kb of DNA a few bases 3' to the junction between the transmembrane and cytoplasmic domains. The

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junctions of the insertion site do not show obvious relationships to splice donor and acceptor sequences. Comparison of the human and murine peptide sequences showed that the peptide sequence diverges at the end of the transmembrane domain, before the nucleotide sequence diverges, suggesting the existence of a selective pressure favoring the creation of a different cytoplasmic domain.

RNA blot analysis showed that myeloid but not lymphocytic cell lines expressed RNA homologous to the CDw32 probe. DNA blot analysis showed multiple bands consistent with the existence of a small multigene family.

Example VI Isolation and Molecular Cloning of Two cDNA Clones Encoding the B Lymphocyte-specific CD20 (B1, Bp35) Antigen

Recent studies suggest that the pan B cell antigen CD20 (B1, Bp35) plays an important role in B cell activation. Monoclonal antibodies (mAb) to CD20 induce different cellular responses depending on the antibody used and the stage of differentiation or activation of the target B cells. The monoclonal antibody 1F5 activates resting B cells by initiating the transition from the G₀ to the G₁ phase of the cell cycle, and induces dense tonsillar B cells to proliferate (Clark et al., Proc. Natl. Acad. Sci USA 82:1766 (1985); Clark and Shu, J. Immunol. 138:720 (1987)). However, 1F5 does not induce an increase in cytoplasmic free calcium and does not induce circulating B cells to proliferate (Rabinovitch et al, In: Leukocyte Typing III (McMichael, Ed.), p. 435, Oxford University Press (1987)). Other anti-CD20 mAbs, such as B1, have been shown to block B cell activation (Tedder et al., J. Immunol. 135:973 (1985)) and both 1F5 and B1 can inhibit B cell differentiation (Golay et al., J. Immunol. 135:3795 (1985)). Recently it has been suggested that phosphorylation and internalization of CD20 may be necessary steps for B cell entry into the G₁ phase of the cell cycle (Valentine et al., In: Leukocyte Typing III (McMichael, Ed.), p. 440, Oxford University Press (1987)). In the present example,

two CD20 cDNA clones were isolated and expressed using the methods of the present invention.

Preparation of cDNA Library and Recovery of cDNA Clones by Panning

5 Poly (A)⁺ RNA was prepared from the human Burkitt cell line Daudi by oligo (dT) cellulose chromatography of total RNA isolated by procedures described herein. cDNA preparation and expression library construction were carried out as described.

10 Anti CD20 mAbs 1F5, 2H7, B1, L27, G28-2, 93-1B3, B-C1, and NU-B2 were obtained from the International Leukocyte Typing Workshop (Valentine et al., In: Leukocyte Typing III (McMichael, Ed.), p. 440, Oxford University Press (1987)). Purified mAbs were used at a concentration of 1 ug/ml and ascites were used at a dilution of 1:1000. Panning was done according to the present method. In the first round of screening, eight 10 cm dishes of 50% confluent COS cells were transfected by the DEAE-Dextran method. Subsequent screening cycles were performed by spheroplast fusion.

Immunoprecipitation, Sequencing, RNA and DNA Blot Hybridization

25 B cell lines CESS and Daudi were metabolically labeled with ³⁵S-methionine and ³⁵S-cysteine for 6h at 37°C. COS cells transfected by the DEAE-Dextran method were similarly labeled 36 hours post-transfection. The labeled cells were incubated with the B1 mAb (Coulter) at 4°C for 1h, washed in PBS, and lysed with 0.5% NP-40, 0.1% SDS, 0.05% deoxycholate and 1 mM PMSF in PBS. After centrifuging (13000xg, 5 min.), the lysate was incubated with fixed S. aureus cells (Calbiochem) for 1 hr at 4°C. The S. aureus cells were pelleted, washed 5 times with 1%NP-40/PBS, eluted and electrophoresed through 12.5% polyacrylamide gels.

300
700
A62
DNA and RNA blot analysis and hybridization probe preparation were carried out as described. Sequencing was done by the method of Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977)). The nucleotide sequence of the CD20.4 cDNA is represented in Figure 10.

Two cDNA clones, bearing inserts of 1.5 (CD20.4) and 1.0 kb (CD20.6), were isolated from a Daudi cell DNA library by panning with a panel of mAbs against CD20. COS cells transfected with either clone reacted with all members of the panel of antibodies. Immunoprecipitation of the cDNA-encloded protein from transfected COS cells showed two bands of 32 and 30 kd reminiscent of the 37 and 35 kd bands observed in different B cell subsets and lines (Valentine et al., "Structure and Function of the B Cell Specific 35-37 kDa CD20 Protein," In: Leukocyte Typing III, A. McMichael et al., eds., Oxford University Press, p. 440 (1987)). It has been the experience of the present inventors that the molecular masses of surface antigens expressed in COS cells are consistently smaller than those of their native counterparts. This may be due to differences in glycosylation.

Both cDNA clones have the same coding sequence, and differ only in the 3' untranslated region. The insert in clone CD20.6 has a short polyA tail and lacks a consensus polyadenylation signal, while the insert in CD20.4 lacks a polyA tail and extends 431bp beyond the 3' terminus in CD20.6 (Fig. 10A).

RNA blot analysis showed that three transcripts of 3.8, 3.0 and 1.5 kb were present in B cells but absent from other cell types, in agreement with the known pattern of antibody reactivity (Clark et al., Proc. Natl. Acad. Sci. USA 82:1766 (1985); Clark et al, J. Immunol. 138:720 (1987); Tedder et al., J. Immunol. 135:973 (1985); Golay et al., J. Immunol. 135:3795 (1985)). It appears likely that the CD20.6 clone is derived from the 1.5 kb transcript or possibly from an even shorter, undetectable species. Because the CD20.4 clone lacks a poly(A)⁺ tail, its source cannot be inferred at present.

DNA blot analysis showed that the CD20 genomic sequences are not rearranged during development and are not amplified in the cell lines examined. A restriction fragment length polymorphism was observed in a DNA sample obtained from placenta.

203
5 The amino acid sequence predicted by the cDNA contains 297 residues and has a molecular mass of 33,097 daltons. The sequence contains three major hydrophobic stretches involving residues 51-103, 117-141 and 183-203 (Fig.10). Two other notable characteristics are the absence of an amino-terminal signal peptide and the presence of a highly charged carboxy-terminal domain. A polyclonal anti-CD20 antibody that recognized the last 18 residues of the carboxy-terminus reacts with lysates of cells expressing CD20 but not with intact cells, suggesting that the CD20 carboxy terminus is located within the cytoplasm. Since there is no amino-terminal signal peptide, it is likely that the amino-terminus is also intracellular, and that the first hydrophobic region acts as an internal membrane insertion signal (Zerial et al., EMBO J. 5:1543 (1986)). The first hydrophobic region is composed of 53 residues and is therefore long enough to span the membrane twice if organized as an alpha helix. Because there are two remaining hydrophobic regions, the intracellular localization of the carboxy-terminus requires that the first hydrophobic domain exit the membrane on the side. Alternatively, the carboxy-terminal antibody may only recognize epitopes exposed by detergent treatment allowing the carboxy-terminus to be extracellular and forcing the first hydrophobic domain to exit the membrane on the extracellular side. The sequence contains 2 potential N-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa cannot be Pro (Bause, Biochem. J. 209:331 (1983)) at positions 9 and 293, but neither of these is expected to be used if located in intracellular domains of the molecule. The difference in molecular mass between CD20 expressed on COS cells and on B cells is therefore presumably due to O-linked glycosylation, although other forms of post-translational modification are not excluded. If the carboxy-terminus is intracellular, the only extracellular domain would lie between residues 142 and 182. This region is rich in serine and threonine residues which might support O-glycosylation.

35 The observation of two protein species in COS cells cannot be explained by alternate splice formation because the cDNA

sequence does not contain any promising splice donor or acceptor sequences (Shapiro et al., Nucl. Acids Res. 15:7155 (1987)). A difference in glycosylation or alternate translational initiation site selection may account for the two species observed.

5 Initiation at either the first or the second ATG gives protein molecular masses of 33.1 and 30.8 kd respectively, in good agreement with the sizes observed in COS cells. Neither ATG is embedded in the consensus sequence proposed by Kozak (Nucl. Acids Res. 12:857 (1984)). Use of alternate initiation sites has been

10 reported for several proteins (Kozak, Nucl. Acids Res. 12:857 (1984)).

Comparison of the peptide sequence with the sequences in the National Biomedical Research Foundation database showed no significant homology by the FASTP rapid sequence alignment algorithm. Because the bulk of the protein appears to be confined to the interior of the membrane and the cell, it seems plausible that it may play a role in transducing signals from other transmembrane proteins to the cell interior. Consistent with this role is the relatively hydrophilic nature of the hydrophobic regions which might allow hydrogen bond interactions with the transmembrane portions of other proteins.

Example VII Isolation and Molecular Cloning of ICAM, An Adhesion Ligand of LFA-1

Antigen-specific cell contacts in the immune system are strengthened by antigen-non-specific interactions mediated in part by lymphocyte function associated or LFA antigens (Springer, T.A., et al., Annu. Rev. Immunol. 5:223-252 (1987); Anderson, D.C., et al., Annu. Rev. Medicine 5:175-194 (1987)). The LFA-1 antigen, a major receptor of T cells, B cells and granulocytes (Rothlein, R., et al., Exp. Med. 163:1132-1149 (1987)), is involved in cytolytic conjugate formation, antibody-dependent killing by NK cells and granulocytes, and helper T cell interactions. LFA-1 has been placed in the integrin family of cell surface receptors by virtue of the high sequence similarity between the LFA-1 and integrin beta chains (Kishimoto, T.K., et al., Cell 48:681-690 (1987); Hynes, R.O. Cell 48:549-554 (1987)).

The adhesion ligands of the integrin family are glycoproteins bearing the Arg-Gly-Asp (RGD) sequence motif, e.g., fibronectin, fibrinogen, vitronectin and von Willebrand factor (Ruoslahti, E., et al., Cell 44:517-518 (1987)).

5 In this example, the Intercellular Adhesion Molecule-1 (ICAM-1), a ligand for LFA-1 (Rothlein, R., et al., J. Immunol. 137:1270-1275 (1986); Dustin, M.L., et al., J. Immunol. 137:245-254 (1986)), was cloned according to the methods of the present invention. ICAM contains no RGD motifs, and instead is
10 homologous to the neural cell adhesion molecule NCAM (Cunningham, B.A., et al., Science 236:799-806 (1987); Barthels, D., et al., EMBO J. 6:907-914 (1987)). COS cells transfected with the ICAM cDNA clone bind myeloid cells by a specific interaction which can be blocked by monoclonal antibodies directed against either LFA-1 or ICAM-1.

A cDNA library was constructed using RNA prepared from HL60 cells induced with phorbol myristyl acetate (PMA). The library was transfected into COS cells and cells expressing surface antigens were recovered according to the methods of the present invention by panning with the anti-ICAM monoclonal antibodies (mAbs) 8F5 and 84H10 (McMichael, A.J., et al., eds., Leukocyte Typing III. White Cell Differentiation Antigens, Oxford University Press (1987)). Episomal DNA was recovered from the
25 panned cells and the expression-panning cycle repeated a further 2 times to obtain a cDNA clone designated pICAM-1.

COS cells transfected with pICAM-1 gave positive surface immunofluorescence reactions with three anti-ICAM-1 antibodies: 8F5; 84H10; and RR-1. Immunoprecipitation of pICAM-1-transfected COS cells with the mAb 84H10 gave a band of molecular mass 100
30 kd. 30). A slightly larger protein of 110 kd was precipitated from HL60 cells induced for 48 hours with either phorbol myristyl acetate (PMA), gamma-interferon (gammaIFN), tumour necrosis factor (TNF), or interleukin-1 beta (IL-1 beta), but was absent from uninduced cells. The smaller molecular mass of ICAM-1

expressed in COS cells is consistent with the lower molecular masses observed for other surface antigens expressed in COS cells.

RNA blot analysis showed 2 species of 3.2 kb and 1.9 kb present in HL60 cells stimulated with either PMA, gamma IFN, TNF or IL-1 gamma, but absent in uninduced cells. Thus, the expression of ICAM-1 is regulated by a number of cytokines, apparently at the level of transcription. Similar species were present in B cells (JY and Raji), T cells (Peer and T blasts) and Lymphokine Activated Killer cells (LAK). The structure of these ICAM-1 transcripts and their relationship to the pICAM-1 cDNA remains to be established. Blot hybridization of genomic DNA from placenta revealed a pattern consistent with a single copy gene.

To investigate whether pICAM-1 encodes a functional cell adhesion molecule, COS cells expressing ICAM-1 were tested for their ability to bind HL60 cells. After 30 minutes at 37°C in the presence of Mg^{2+} , HL60 cells strongly adhered to the ICAM-expressing COS cells, but not to mock transfected cells. The specificity of this adhesion was demonstrated by preincubating the ICAM-1 expressing COS cells with mAb 84H10. All HL60 binding was abolished under these conditions. An isotype matched monoclonal antibody, W6/32, which recognizes a monomorphic HLA-ABC related determinant of approximately equal abundance to ICAM-1 on transfected COS cells, had no effect on the adhesion. Similarly, preincubation of the HL60 cells with either 84H10 or W6/32 did not inhibit binding.

To determine if LFA-1 was acting as the receptor for ICAM-1 in this system, HL60 cells were pretreated with antibodies against the beta chain of LFA-1 (CD18 (McMichael, A.J., et al., eds., Leukocyte Typing III. White Cell Differentiation Antigens, Oxford University Press (1987))) and then subjected to the binding assay. All adhesion to ICAM-expressing COS cells was blocked. Pretreatment of COS cells with the CD18 antibodies had

no effect on the adhesion. This provides direct evidence that ICAM-1 is indeed acting as an adhesion ligand for LFA-1.

5 The sequence of the pICAM-1 cDNA insert consists of 1846 nucleotides (Fig. 11). The predicted peptide sequence of 532 residues has the typical features of a transmembrane protein including a putative signal sequence, which may be cleaved between glycine-25 and asparagine-26 (von Heijne, G., Nucl. Acids Res. 14:4683-4690 (1986)), and a single 25 residue membrane-spanning domain terminating in a short, highly charged cytoplasmic domain. The extracellular domain contains seven potential N-linked glycosylation sites which could adequately explain the difference in size between the deglycosylated precursor (55 kd) and the final product (90-115 kd) (Dustin, M.L., et al., J. Immunol. 137:245-254 (1986)). Differential use of these putative glycosylation sites could also explain the heterogeneous molecular mass of ICAM-1 observed in different cell types (Dustin, M.L., et al., J. Immunol. 137:245-254 (1986)).

20 LFA-1 is a member of the integrin family of cell surface receptors (Kishimoto, T.K., et al., Cell 48:681-690 (1987); Hynes, R.O., Cell 48:549-554 (1987)). The tripeptide motif Arg-Gly-Asp (RGD) is a common feature of the ligands for this family, e.g., fibronectin, fibrinogen, vitronectin and von Willebrand factor, and is crucial for ligand-receptor interaction (Ruoslahti, E, et al., Cell 44:517-518 (1987)). However, ICAM-1 contains no RGD motifs, bearing instead a single RGE sequence at position 152. A search of the National Biomedical Research Foundation (Dayhoff, M.O., et al., Methods Enzymol. 91:524-545 (1983)) (NBRF) database revealed no significant similarities to other proteins. However, a comparison to a laboratory database containing recently published surface proteins did reveal a surprising and significant similarity between ICAM-1 and the neural cell adhesion molecule NCAM-1 (Cunningham, B.A., et al., Science 236:799-806 (1987); Barthels, D., et al., EMBO J. 6:907-914 (1987)). The optimal alignment score obtained using the NBRF
35 ALIGN program is 8 standard deviations above the mean score

obtained from 500 random permutations of the sequences. The probability of the spontaneous occurrence of an equal or higher score is approximately 10^{-9} .

Using a database of known immunoglobulin related sequences, it has been shown that ICAM-1 may be divided into five Ig domains (28-112, 115-206, 217-310, 312-391, and 399-477) each of which shows significant similarity with other members of the Ig superfamily (Williams, A.F., Immunol. Today 8:298-3-3 (1987)). For example, domain I is similar to CD3 while domains IV and V are similar to domains of myelin associated glycoprotein (Arguint, M., et al., Proc. Natl. Acad. Sci. USA 84:600-604 (1987)) and carcinoembryonic antigen (Beauchemin, N., et al., Mol. Cell. Biol. 7:3221-3230 (1987)). All five Ig domains of NCAM align with the Ig segments in ICAM, and the principal contribution to the similarity comes from domains II and III of ICAM. Finally, the T cell-specific adhesion molecule CD2 shows roughly the same similarity to NCAM as does ICAM, but ICAM and CD2 are only weakly related. Thus, some precursor of NCAM is ancestral to both ICAM and CD2.

Through its cell adhesion to LFA-1, ICAM can mediate migration of lymphocytes into areas of inflammation. Inhibiting such migration by blocking ICAM binding to LFA-1 could reduce or inhibit inflammation. Such inhibition could be affected by small organic molecules, i.e., drugs, identified in an ICAM streaming assay. Fusion proteins composed of the extracellular domain of ICAM and IgG molecules are suitable for identifying such inhibitors. Likewise, compounds that interfere with ICAM binding to Rhinovirus or Plasmodium falciparum can be identified by analogous methods.

The Applicants have constructed fusion proteins consisting of the Ig domains of the ICAM-1 extracellular domain, which are useful in streaming assays. The CH2 and CH3 domains of IgG1 were fused to extracellular Ig domain 1, domains 1 and 2, domains 1-3, domains 1-4, and domains 1-5 of ICAM-1. The corresponding clones

have been designated ICAM1-E γ 1, ICAM1-E γ 2, ICAM1-E γ 3, ICAM1-E γ 4 and ICAM1-E γ 5. The first two N-terminal domains (amino acids 28-112 and 115-206, respectively) are believed to be most useful in a binding assay to identify compounds that inhibit ICAM binding to another ligand.

Soluble ICAM can also be used directly to interfere with the binding of cellular ICAM to Rhinovirus or Plasmodium, thereby inhibiting the infection. Soluble ICAM includes the extracellular domain of ICAM, or functional derivatives thereof, in truncated form or fused to a soluble protein such as Ig.

For the purpose of the invention as it relates to ICAM-1 protein, the term "functional derivatives" includes polypeptides that have at least about 80% amino acid identity to the entire disclosed ICAM-1 amino acid sequence, its intracellular domain, its extracellular domain (amino acids 1-477) or any of immunoglobulin domains 1-5 therein, or the sequence comprising the LFA-1 binding site, and that have a binding affinity to a ligand of ICAM-1 such as LFA-1 that is at least about 30% as that of the disclosed sequence. Such polypeptides may optionally be included as part of a larger protein. Increasingly preferred are amino acid identities that increase integrally, i.e., at least about 81%, 82%, 83%, etc. Binding affinity of ICAM-1 to a ligand such as LFA-1 can be determined by methods known in the art. Increasingly preferred are binding affinities that increase in increments of 10%, i.e., at least about 40%, 50%, etc., of that of the disclosed sequence.

A nucleotide sequence is a "functional derivative" of ICAM-1 if it encodes the disclosed ICAM-1 amino acid sequence or a functional derivative thereof.

In designing functional derivatives of ICAM-1 protein, amino acids or regions known to be important to the binding of ICAM-1 to a ligand such as LFA-1 should be highly conserved. Publications relating to binding of ICAM-1 to LFA-1 include:

Hedman, H. & Ludman, E. (1991) J. Immunol. 149:2295-9; Dang, L & Rock, K. (1991) J. Immunol. 146:3273-9; Ross, L et al. (1992) J. Biol.Chem. 267:8537-43; Hibbs, M. et al. (1991) Science 251:1611-3; Berendt, A. et al. (1992) Cell 68:71-81; Ockenhouse, C. et al. (1992) Cell 68:63-9; Diamond, M. et al. (1991) Cell 65:961-71; Cabanas, C. & Hogg, N. (1991) FEBS Lett 292:284-8; and Staunton, D. et al. (1990) Cell 61:243-54 (errata at Cell (1990) 61:1157 and Cell (1991) 667:1312).

Additional publications relating to function of ICAM-1 include: Salkind, A. et al. (1991) J. Clin. Invest. 88:505-11; Fischer, H. et al. (1992) J. Immunol. 148:1993-8; Carlow, D. et al. (1992) J. Immunol. 148:1595-606; Rothlein, R. et al. (1991) J. Immunol. 147:3788; Fine, J. & Kruisbeek, A. (1991) J. Immunol. 147:2852-9; Tang, A. & Udey M. (1991) J. Immunol. 146:3347-55; Symington, F. & Santos, E. (1991) J. Immunol. 146:2169-75; Rosenstein, Y. et al. (1991) Nature 354:233-5; Fujiota, H. et al. (1991) Biochem. Biophys. Res. Commun. 177:664-72; Nambu, M. et al. (1992) Cell Immunol. 143:335-47; Sanders, V. & Vitetta, E. (1991) Cell Immunol. 132:45-55; Diamond, M. et al. (1990) J. Cell Biol. 111:3129-39; Maraskovsky, E. et al. (1992) Int. Immunol. 4:475-85; Padros, M. et al. (1992) Clin. Exp. Immunol. 88:329-34; and Van Seventer, G. et al. (1991) Eur. J. Immunol. 21:1711-18.

In particular, Piela-Smith, T. et al. (1992) J. Immunol. 148:1375-81; Staunton, D. et al. (1990) Cell 61:243-54 (errata at Cell (1990) 61:1157 and Cell (1991) 667:1312); Staunton, D. et al. (1989) Cell 56:849-53; Greve, J. et al. (1989) Cell 56:839-47; Staunton, D. et al. (1992) J. Immunol. 148:3271-4; Register, R. et al. (1991) J. Virol. 65:6589-96; Greve, J. et al. (1991) J. Virol. 65:6015-23; and Lineberger, D. et al. (1992) Virus Res. 24:173-186, discuss binding of ICAM-1 to rhinovirus. Oppenheimer-Marks, N. et al. (1991) J. Immunol. 147:2913-21; and Perry, M. et al. (1992) Cell Tissue Res. 268:317-26; describe the role of ICAM-1 in transendothelial migration, and Dustin, M. et al. (1992) J. Immunol. 148:2654-63, describe B cell migration on ICAM-1 coated substrates. Webb, D. et al. (1991) J. Immunol.

146:3682-6, describe the role of ICAM-1 in monocyte invasion of tumors. Berendt, A. et al. (1992) Cell 68:71-81; Ockenhouse, C. et al. (1992) Cell 68:63-9; and Ockenhouse, C. et al. (1991) J. Infect. Dis. 164:163-169, discuss ICAM-1 in its role as a receptor for Plasmodium falciparum. Gruber, M. et al. (1991) AIDS Res. Hum. Retroviruses 7:45-53, discuss the role of ICAM-1 in HIV syncytia formation. Dohlsten, M. et al. (1991) Eur. J. Immunol. 21:131-5, discuss the role of ICAM-1 in staphylococcal enterotoxin-mediated cytotoxicity.

Example VIII Isolation and Molecular Cloning of the Human CD19, CD20, CDw32a, CDw32b and CD40 Antigens

The rapid immunoselection cloning method of the present invention was applied to isolate and clone the CD19, CD20, CDw32a, CDw32b, and CD40 antigens. The nucleotide sequence of CD19 is shown in Figure 12. The nucleotide sequence of CD20 is shown in Figure 13. The nucleotide sequence of CDw32a is shown in Figure 15. The nucleotide sequence of CDw32b is shown in Figure 16. The nucleotide sequence of CD40 is shown in Figure 17.

Example IX Cloning, Sequence and Expression of CD36

To isolate a cDNA clone encoding CD36, a human placenta cDNA (Simmons and Seed (1988) Nature 333:568-570) was transferred into COS cells using DEAE-Dextran as a facilitator (Example I, supra). 48 hours posttransfection the cells were detached from the dishes without trypsin, incubated with monoclonal anti-CD36 antibodies 5F1 (Bernstein et al. (1982) J. Immunol. 128:876-881) (Andrews et al. (1984) J. Immunol. 128:398-404) and panned on dishes coated with goat anti-mouse immunoglobulin antibodies. Nonadherent cells were removed by gentle washing, the adherent cells were lysed, and episomal plasmids recovered from the cells were purified and transformed into E. coli. After two similar rounds of enrichment following spheroplast fusion, plasmid DNAs recovered from 11 out of 12 randomly chosen colonies were found to direct the appearance of CD36 determinants in transfected COS cells.

Two of the clones were chosen at random for further analysis. Both bore inserts of about 1.9 kb, and showed identical restriction enzyme fragment patterns. COS cells transfected with either of these clones reacted with monoclonal antibodies 5F1 and F13, and with OKM5 (Ortho, Raritan, NJ). Immunoprecipitation of transfected cells with a pool of anti-CD36 antibodies revealed the presence of an 83 kd molecule present on transfected COS cells and C32 melanoma cells, and absent from control (CD25-transfected) COS cells. A high molecular weight species, possibly dimeric CD36, was immunoprecipitated from the transfected COS cell lysate, but not from the C32 cell lysate. The nucleotide sequence is given in Table 1. In Table 1, the nucleotide sequence numbering is shown in the left margin at the beginning of each line. The deduced amino acid sequence is shown as single letter code underneath the beginning of each coding nucleotide triplet, with the initiation methionine indicated by the number 1 above the initiator codon. The potential sites of N-linked glycosylation in the derived amino acid sequence are underlined by a single dashed line. The putative transmembrane domain is double-underlined. Although two consensus polyadenylation (AATAAA) motifs are found in the cDNA, there is no poly(A) tail, and none of the RNA species observed by blot hybridization are short enough to correspond to polyadenylation at these sites, assuming that the transcripts bear the same approximate 5' end as observed in the clone. The presumed initiation codon is not the first ATG found in the clone, but the previous two are closely followed by in-frame termination codons. The predicted initiator methionine is followed by a short hydrophobic region resembling a secretory signal sequence for which, however, no clear identification of the cleavage site can be made. The recent determination of Table 1 the amino terminal 36 amino acid sequence (Tandon et al. J. Biol. Chem. 264:7570-7575 (1989)) indicates that the mature polypeptide begins at the amino acid residue immediately following the initiator methionine. It is not clear whether the single Arg residue preceding the hydrophobic region would be sufficient to allow amino terminal membrane anchoring. The resulting polypeptide

possesses 471 residues with a predicted molecular weight of about 53 kd. The proposed extracellular domain is followed by 27 predominantly hydrophobic residues corresponding to a transmembrane domain, and 6 residues (of which 3 are basic) corresponding to an attenuated intracellular domain. The presence of 10 potential N-linked glycosylation sites appears sufficient to account for the discrepancy in molecular mass between the predicted polypeptide and the 83 kd species found by immunoprecipitation. No significant homology was detected following comparison of the entire sequence to various databases of known proteins. However some internal structure was apparent. All of the cysteine residues in the extracellular domain are confined to a domain defined by residues 937 to 1209 of the nucleotide sequence. Taking the cysteine placement as a guide, the extracellular domain could be divided into three segments, in which two domains without cysteine preceded and followed the cysteine rich segment. However sequence comparisons with these segments did not show any significant relatedness to other molecules in existing databases, nor, in particular, to thrombospondin.

The CD36 protein was purified by immunoprecipitation. Since the rapid immunoselection cloning method depended upon expression of transfected COS cells, it follows that the same cell lines from which CD36 cDNA was cloned could also be used as a source of the expressed protein.

C32 melanoma cells, CD36 transfected cells COS cells, and CD25 (control) transfected COS cells were surface labelled with Na^{125}I and lysed in a phosphate buffered saline solution containing 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40 and 0.1% sodium dodecyl sulfate. Anti-CD36 monoclonal antibodies were added, and allowed to absorb to the lysate for 12 hours at 4°C, after which goat anti-mouse Immunoglobulin beads (Cappel)

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were added, mixed for two hours, and washed as described (Clark and Einfeld J. Immunol. 135:155-167 (1986)). Larger amounts of protein can also be obtained in purified form from a transfected COS cell lysate by an immunoaffinity column purification. Other
5 antibodies to CD36 may be obtained, using CD36 protein, expressed and/or purified as described, as immunogen.

10 CD36 has been identified as a binding site for cytoadherence of Plasmodium falciparum parasitized erythrocytes, by the inventors herein and by Ockenhouse, C.D. et al. (1989) Science 243:1469-1741. Cytoadhesion of parasitized erythrocytes has been shown to be blocked by monoclonal antibodies to CD36. Incubation of infected erythrocytes with COS cells transfected with a CD36 cDNA showed pronounced cytoadherence. The ability of P. falciparum parasitized erythrocytes to evade splenic clearance by adherence to peripheral vascular beds is thought to play an important role in the pathogenicity of Falciparum malaria and to contribute to the lethal syndrome of cerebral malaria by causing occlusion of the small vessels of the brain. Therefore the cDNA and purified protein of the present invention are useful for providing sufficient purified CD36 to make therapeutic monoclonal antibodies.

Example X Isolation and Cloning of Three cDNA Clones Encoding Macrophage-Specific FcRI

25 Three independent cDNA clones (designated p135, p90 and p98/X2) encoding human FcRI were isolated by the rapid immunoselection cloning method of the present invention from a cDNA library expressed in COS cells. (See also Allen, J.M. and Seed B., Science 243:378-381 (1989)). The cDNA library was
30 constructed from polyadenylated RNA obtained from cells of a single patient undergoing extracorporeal interleukin-2 induction therapy. Expression of the three cDNAs in COS cells gave rise to IgG binding of the appropriate affinity and subtype specificity. DNA sequence analysis revealed that the cDNAs encode similar type I integral membrane proteins with 3
35 extracellular immunoglobulin domains. The intracellular domain of p98/X2 diverges from that of the other two cDNAs. A composite

sequence of the three cDNAs is shown in Table 2 with the nucleotide differences of the p89/X2 or p90 clones shown respectively below or above the p135 sequence. Dashes denote gaps and no residues are shown above or below where the sequences are identical. The p90 cDNA has the shortest 5' untranslated region, 7 additional residues between the polyadenylation motif and the poly A tract, and 2 polymorphisms in the coding region. The p98/X2 cDNA has the longest 5' untranslated region, 1 polymorphism in the coding sequence, and diverges from the other two cDNAs at residue 1051, becoming a complex pattern of repeats of upstream sequences. The p98/X2 clone lacks a polyadenylation site.

The FcRI protein from each of the three clones was purified from the respective COS cell lines which expressed them, by immuno-adsorption to IgG-agarose. (See Stengelin S. et al., EMBO J. 7:1053 (1988)). Gel electrophoresis of purified proteins showed a single species from p135 and p90 COS cells, relative molecular size 70 kd. Cells transfected with p98/X2 expressed a protein of 67 kd. A slightly larger protein of 75 kd was adsorbed from untreated and interferon-gamma-treated U937 promonocyte cells. The smaller mass observed in COS cells is consistent with the reduced masses observed from Table 2 other surface antigens expressed in COS cells, see e.g., Example IX.

The predicted polypeptide sequences show the typical features of a type I integral membrane protein, and include a short hydrophobic signal sequence, a single 21-residue hydrophobic membrane-spanning domain, and a short, highly charged cytoplasmic domain (Fig. 4). The extracellular portion contains six potential N-linked glycosylation sites and six Cys residues distributed among three C2 set Ig-related domains.

FcRI is a high-affinity receptor for the Fc portion of IgG, normally located on the cell surfaces of macrophages. The ability to interfere with such bonding, or to cause it to occur on surfaces other than macrophages, is useful in therapy. For

87

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example, a fusion protein of FcRI and a receptor ligand will be helpful to increase the potency of antibodies in therapy.

Example XI Isolation and Cloning of cDNA Encoding T-Lymphocyte TLiSA Antigen

5 A cDNA clone encoding TLiSA1 was obtained from a human T-cell cDNA library transferred into COS cell as described and subjected to the rapid immunoselection cloning method of the invention. A monoclonal antibody ACT-T-SET TLiSA1 (T-Cell Sciences Corp., Cambridge, Massachusetts) was used to detect
10 transfected COS cells expressing the cloned cDNA, by positive indirect immunofluorescence. The positive plasmid contained in a 1.7 kb insert.

TLiSA protein was isolated by immunoprecipitation, as described supra, Example IX. The protein had a molecular weight of about 50 kd, as measured by gel electrophoresis.

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The nucleotide sequence of the cDNA was determined by dideoxynucleotide chain termination as described, supra. The sequence of 1714 residues is given in Table 3, together with the deduced amino acid sequence shown in single letter code under the first nucleotide of each coding triplet. The ATG encoding the presumed initiator methionine is followed by a short hydrophobic region consistent with a secretory signal sequences, the most likely excision site being 19 residues into the open reading frame. The resulting polypeptide, if not further processed, would
25 possess 317 residues with a predicted molecular weight of about 36 kd. The proposed extracellular domain is followed by 25 predominantly hydrophobic residues corresponding to the intracellular domain. (Table 3, double underlined.) The
30 presence of 9 potential N linked glycosylation sites (Table 3, single dashed lines) appears sufficient to account for the discrepancy in molecular mass between the predicted polypeptide and the 50 kd species found by immunoprecipitation.

TLISA is involved in mediating IL-2 induced differentiation of T-cells into cytolytic forms. Antibodies to TLISA are useful to prevent IL-2 stimulated T cell differentiation, and to modulate adverse effects of IL-2 in therapy.

Example XII The Isolation and Molecular Cloning of cDNA Encoding for B lymphocyte-specific CD22 Antigen

To isolate a CD22 cDNA, an expression library was constructed from the Burkitt lymphoma cell line Daudi, introduced into COS cells by the DEAE-Dextran method described supra, and subjected to three rounds of panning and re-introduction into E. coli as described in Seed and Aruffo, Proc. Natl. Acad. Sci. USA 84:3365-3369 (1987) and Aruffo and Seed, Proc Natl. Acad. Sci. USA 84:8573-8577 (1987). Of 16 plasmids picked after the third round, two tested positive for CD22 expression by indirect immunofluorescence in COS cells. Of the five carbohydrate-related epitopes, A, B, C, D and E, recognized by anti-CD22 monoclonal antibodies, only epitopes A and D were expressed in COS cells.

Immunoprecipitation of CD22 from transfected COS cells yielded a single band corresponding to a molecular mass of 110 kd, smaller than the 135 kd species obtained from Burkitt lymphoma Raji cells. The difference in mass may be related to differences in glycosylation. Since the immunogenic epitopes of CD22 are carbohydrate-related, these differences might account for the absence of epitopes B, C and E.

RNA blot hybridization analysis has revealed the presence of a major 3 kb RNA species and 4 minor species of 2.6, 2.3, 2.0 and 1.5 kb in several B cell lines. RNA encoding CD22 has not been found in several T cell lines, including peripheral blood T cells, the T cell leukemia Jurkat, the myeloid leukemia lines HL60 and U937 and the hepatoma HepG2.

TABLE 3

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TABLE 4

1	ACGGGGAAC AGCTTGCAC CCAGACACGA CACCATGCAT CTCCTCGGCC CTTGGCTCCT GCTCCTGGTT CTAGATAACT	M H L L G P W L L L L V L E Y L
81	TGGCTTTCTC TGACTCAAGT AAATGGTTT TTGAGCACCC TGAAACCCTC TACGCCCTGG AGGGGGCTG CGTCTGCATC	A F S D S S K W V F E H P E T L Y A W E G A C V W I
161	CCCTGCACCT ACAGAGCCCT AGATGGTGAC CTGGAAGCT TCATCCTGTT CCACAATCCT GAGTATAACA AGAACACCTC	P C T Y R A L D G D L E S F I L F H N P E Y N K N T S
241	GAGTTTGAT GGGACAGAC TCTATGAAG CACAAGGAT GGAAGTTT CTCTGACA GAAAGGCTG CAATTCCTGG	K F D G T R L Y E S T K D G K V P S E Q K R V Q F L G
321	GAGACAGAA TTAGAATGTC ACACTGAGTA TCCACCCGGT GCACCTCAAT GACAGTGTG AGCTGGGGCT GAGATGGAG	D K N K N C T L S I H P V H L N D S G Q L G L R M E
401	TCCAGACTG AGAATGGAT GGAACGAATA CACCTCAATG TCTCTGAAG GCCTTTTCCA CCTCATATCC AGCTCCCTCC	S K T E K W M E R I H L N V S E R P F P P H I Q L P P
481	AGAAATTCAA GAGTCCCAGG AAGTCACTCT GACCTGCTTG CTGAATTTCT CTTGCTATGG GTATCCGATC CAATTCAGT	E I Q E S Q E V T L T C L L N F S C Y G Y P I Q L Q W
561	GGCTCCTAGA GGGGTTCCA ATGAGCAGG CTGCTGTGAC CTGCACCTCC TTGACCATCA AGTCTGTCTT CACCCGGAGC	L L E G V P M R Q A A V T S T S L T I K S V F T R S
641	GAGCTCAAGT TCTCCCCACA GTGAGTCAAC CATGGGAAAT TTGTGACCTG CCAGCTTCAG GATGAGATG GGAAGTTCT	E L K F S P Q W S H H G K I V T C Q L Q D A D G K F L
721	CTCCAATGAC ACGGTGCAGC TGAACGTGA GCATCCTCCC AAGAGGTGA CCACAGTGAT TCAAAACCCC ATGCCGATTC	S N D T V Q L N V K H P P K K V T T V I Q N P M P I R
801	GAGAAGAGA CACAGTGACC CTTTCTGTA ACTACAAATTC CAGTAACCCC AGTGTAAACC GGTATGAATG GAAACCCAT	E G D T V T L S C N Y N S S N P S V T R Y E W K P H
881	GGGCGCTGG AGGAGCCATC GCTTGGGCTG CTGAAGATCC AAAACGTTGG CTGGACACAC ACAACCATCG CCTGCGCAGC	G A W E E P S L G V L K I Q N V G W D N T T I A C A A
961	TTGTAAATAGT TGGTGTCTCT GGGCTTCCC TGTCGCCCTG AATGTCCAGT ATGCCCCCG AGACGTGAGG GTCCGGAATA	C N S W C S W A S P V A L N V Q Y A P R D V R V R K I
1041	TCAAGCCCCC TTCCGAGATT CACTCTGAA ACTCGGTGAG CCTCCAAATGT GACTTCTCAA GCAGCCACCC CANAGAGTGC	K P L S E I H S G N S V S L Q C D F S S S H P K E V

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TABLE 4

1121 CAGTCTCTCT GGGAGAAAA TGGCAGGCTT CTGGGAAAG AAAGCCAGCT GAATTTGAC TCCATCTCCC CAGAAGATGC
 Q F F W E K N G R L L G K E S Q L N F D S I S P E D A
 1201 TGGAGTTAC AGCTGCTGGG TGAACAACTC CATAGACAG ACAGCCTCCA AGCCTGGAC ACTTGAGTG CTGATGCAC
 G S Y S C W V N N S I G Q T A S K A W T L E V L Y A P
 1281 CCAGGAGGCT GCGTGTGTC ATGAGCCCGG GGGACCAAGT GATGAGGGG AAGAGTGCA CCCTGACCTG TGAAGCGAC
 R R L R V S M S P G D Q V M E G K S A T L T C E S D
 1361 GCCAACCTC CCGTCTCCA CTACACCTGG TTTGACTGA ATAACCAAG CCTCCCTAC CACAGCCAGA AGCTGAGTT
 A N P P V S H Y T W F D W N N Q S L P Y H S Q K L R L
 1441 GGAGCCGTC AAGTCCAGC ACTCGGCTC CTACTGTGC CAGGGACCA ACAGTGTGG CAGAGGCCGT TCGCCTCTCA
 E P V K V Q H S G A Y W C Q G T N S V G K G R S P L S
 1521 GCACCTCAC CGTCTACTAT AGCCCGAGA CCATCGGCAG GCGAGTGGCT GTGGACTCG GGTCTGCTT CGCCATCTC
 T L T V Y Y S P E T I G R R V A V G L G S C L A I L
 =====
 1601 ATCCTGGCA TCTGTGGCT CAGCTCCAG CGAGTTGA AGAGACACA GAGCCAGAG GGGCTTCAGG AGAATTCAG
 I L A I C G L K L Q R R W K R T Q S Q Q G L Q E N S S
 =====
 1681 CGGCCAGAC TTCTTTGTGA GGAATAAAA GGTAGNAGG GCCCCCTCT CTGAAGGCC CCACTCCCTG GGATGCTACA
 G Q S F F V R N K K V R R A P L S E G P H S L G C Y N
 1761 ATCCATGAT GGAAGATGC ATTAGCTACA CCACCCTCG CTTCCTCGAG ATGACATAC CACGAACTGG AGATGACAG
 P M M E D G I S Y T T L R F P E M N I P R T G D A E
 1841 TCCTCAGAG TGCAGAGACC TCCCCCGAC TCGCATGACA CGGTCACTTA TTGACATTG CACAGCGCC AAGTGGCAC
 S S E M Q R P P P D C D D T V T Y S A L H K R Q V G T
 1921 TATGAGAAC TCAATTCAGA TTTTCAGAA GATGAGGGA TTCAATTAAC AGAGCTGATC CAGTTTGGG TCGGGAGCG
 M R T S F Q I F Q K M R G F I T Q S *
 2001 GCCTCAGCA CAGAAAAAG TGAATAATG GATCTCANA CATTGAACT GAGTGGCTG CAGCAGAGC ACTGGGGCA
 2081 GCGGGGCCA GGAAGTCCC CGAGTTT

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5 DNA blot hybridization of placental DNA gave a simple
pattern consistent with a single copy gene. DNA sequence
analysis by the dideoxy method described supra showed that the
2107 bp insert encoded a polypeptide of 647 amino acids. The
nucleotide and amino acid sequences appear in Table 4. The
initial methionine is followed by 18 predominantly hydrophobic
amino acids resembling a secretory signal sequence. The mature
protein, having a relative molecular weight of 71.1 kd consists
of an extracellular portion of 491 residues, followed by a 19
10 residue membrane-spanning domain (doubly underlined), and an
intracellular domain of 118 amino acids. Ten potential N-linked
glycosylation sites (N-X-S/T, X not equal to P) are found in the
predicted extracellular domain, as well as a large number of
serine and threonine residues which may be sites of O-linked
glycan addition. The abundance of potential glycosylation sites
and the difference in mass between the predicted protein backbone
and the product precipitated from COS cells and B cell lines
suggest that about 50% of the mass of CD22 is contributed by
carbohydrate.

20 The extracellular portion of CD22 consists of five segments
having Ig-like domain organization. The short intercysteine
spacing (63 and 64 residues in domains 1 and 2, and 42 residues
in domains 3-5) suggests that they fold into the 7 strand two
layer beta-sheet structure characteristic of immunoglobulin
constant regions rather than the 9 strand structure of variable
25 regions.

30 Because CD22 has been found to be highly homologous to
myelin associated glycoprotein (MAG), a neuronal cell surface
protein which mediates cell-cell contacts during myelogenesis,
it was postulated that CD22 has a role in B cell adhesion. COS
cells transfected with CD22 cDNA were contacted with erythrocytes
or peripheral blood mononuclear cells and incubated under
conditions which minimized nonspecific interaction. Erythrocyte
and mononuclear cell resetting was observed with CD22-positive

COS cells but not with COS cells transfected with an unrelated cDNA clone.

5 B cell adhesion studies involving anti-epitope monoclonal antibodies have indicated that different epitopes of CD22 may participate in erythrocyte and monocyte adhesion and that different ligands may be recognized on each cell type. B cell adhesion studies also suggest that CD22, in a manner analogous to T cell CD2, CD4 and CD8 adhesion to target cells, may promote recognition by the B cell antigen receptor by intensifying B cell-presenting cell contacts. CD22 has been previously
10 implicated in the transmission of signals synergizing with the antigen receptor (Pezutto et al., J. Immunol. 138:98-103 (1987)) and crosslinking of surface IgM produces an intracellular calcium flux in IgM⁺CD22⁺ but not in IgM⁺CD22⁻ cells (Pezutto et al., J. Immunol. 140:1791-1795 (1988)). These results suggest that, like T cell accessory molecules, CD22 may also participate in the regulation of signal transduction.

The ability to interfere with the binding of CD22 positive B cells with accessory cells, or the ability to cause such binding to occur on surfaces other than lymphocyte cells can be useful in diagnostics and therapy. For example, a fusion protein of CD22 and a receptor ligand fixed to a substrate will be useful in detecting the presence of a particular antigen in body fluids. A soluble form of CD22 can have immunomodulatory activity.

25 Example XIII The Isolation and Molecular Cloning of cDNA Encoding for T Lymphocyte-specific CD27 Antigen

WAB A72
30 A cDNA clone encoding CD27 was obtained from human T lymphocyte cDNA transferred into COS cells and immunoselected by the method of the present invention. RNA was extracted from the mononuclear cells derived from a unit of blood, after four days of culture in medium containing 1 ug/ml phytohemagglutinin (PHA), using guanidium thiocyanate. The total RNA was poly-A selected. cDNA was made and cloned into CDM8, transfected into COS cells and the CD27 cDNA was immunoselected with monoclonal antibodies
35 OKT18a and CLB-9F4 (provided as described in Seed and Aruffo

Cont
A72
Proc. Natl. Acad. Sci. 84:8573-8577 (1987); and Aruffo and Seed
Proc. Natl. Acad. Sci. USA 84:3365-3369 (1987)). The vector
contained a 1.2 kb/cDNA insert.

5 The nucleotide sequence of the cDNA was determined by
dideoxynucleotide chain termination as described, supra. The
sequence of 1203 residues and the deduced amino acid sequence
appear in Table 5. The initiation methionine is indicated by the
number 1 above the initiator codon. The deduced CD27 polypeptide
10 demonstrates the typical features of a type I integral membrane
protein. It begins with a twenty amino acid hydrophobic region
consistent with a secretory signal sequence. This hydrophobic
region is followed by a 171 residue extracellular domain, a 20
residue hydrophobic membrane spanning domain (doubly underlined)
and a 49 amino acid cytoplasmic domain beginning with a
positively charged stop transfer sequence. There is no poly (A)
tail.

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25 The deduced CD27 amino acid sequence is highly homologous
to the B lymphocyte and carcinoma antigen CD40, described supra,
over its entire length. CD27 is also highly homologous to the
the the receptor for nerve growth factor (NGFR) over the
extracellular and transmembrane domains (Stamenkovic et al., EMBO
J. 8:1403-1410 (1989); Johnson et al., Cell 47:545-554 (1989)).
The most conserved structural motif found in these three proteins
is the abundance of cysteines or histidines in the extracellular
25 region. These are often found in pairs separated by two or four
intervening amino acid residues, similar to the arrangement seen
in proteins which use this structure to bind a zinc ion. The
cysteine and histidine rich region is followed by a serine,
threonine and proline rich membrane proximal domain which has
30 been suggested to be the region in which biochemically identified
O-linked glycans are added to NGFR (Johnson et al. (1986), supra;
Grob et al., J. Biol. Chem. 260:8044-8049 (1985)).

Immunoprecipitation of transfected COS cells with anti-CD27
antibodies followed by gel electrophoresis revealed the presence

TABLE 5

1	GGGGTGCNAA	GNAGAGACAG	CAGCGCCCAAG	CTTGAGAGTG	CTAACTCCAG	AGGCCAGCAT	CAGCAACTGG	GCACAGNAAAG
81	GAGCCGCCCTG	GGCAGGGACC	1 M A R P H P W L C V L G T L V G L S A					
161	ACTCCAGCCC	CCNAGAGCTG	CCCAGAGAGG	CACACTGCGG	CTCAGGGA	GCCTGCTGC	CAGATGTGTG	AGCCAGNAA
241	ATTCCCTCTG	AAGGACTGTG	ACCAGCATTAG	AAAGGCTGCT	CAGTGTGATC	CTTGCAATACC	GGGGGTCTCC	TTCTCTCCTG
321	ACCAACCAAC	CCGCCCCCAC	TGTGAGAGCT	GTCCGCACCTG	TAACTCTGGT	CTTCTCGTTC	GCAACTGCAC	CATCACTGCC
401	AATGCTGAGT	GTGCTGTCTG	CAATGGCTGG	CAGTGCAGGG	ACAAGGAGTG	CACCGAGTGT	GATCCTCTTC	CNAACTTC
481	GCTGACCGCT	CGGTGCTCTC	AGGCCCTGAG	CCCAACACCT	CAGCCCCACCC	ACTTACCTTA	TGTCACTGAG	ATGCTGAGG
561	CCAGGACAGC	TGGGCACATG	CAGACTCTGG	CTGACTTCAAG	GCAGCTGCCT	GCCCCGACTC	TCTCTAACCA	CTGGCCACCC
641	CNAAAGTCCC	TGTGCAGCTC	CGATTTTAA	CGCATCTCTG	TGATCTTCTC	TGGAATGTTT	CTTGTTTTCA	CCCTGGCCGG
721	GGCCCTGTTC	CTCCATCAAC	GAAGGAATA	TAGATCAAAC	AAAGGAGAA	GTCCCTGTGA	GCCTGCAGAG	CCTTGTCTGT
801	ACAGCTGCCC	CAGGAGGAG	GAGGGCAGCA	CCATCCCCAT	CCAGAGAGAT	TACCGAANAAC	CGAGCCCTGC	CTGCTCCCC
881	TGAGCCNAGCA	CCTGCGGTAG	CTGCACTACA	GCCCTGGCCT	CCACCCCAAC	CCCGCCGACC	ATCCAAGGGA	GAGTGAGACC
961	TGGCAGCCAC	AACTGCAAGT	CCATCCTCTT	GTGAGGGCCC	TTTCTGTGT	ACACGTGACA	GAGTGCCCTT	TCGAGACTGG
1041	CAGGAGCAG	GACAAATATG	GATGAGGTGG	AGAGTGGGAA	GCAGGAGCCC	AGCCAGCTGC	GCGCGCTGC	AGGAGGGCGG
1121	GGGCTCTGCT	TGTNAGGCAC	ACTTCTGCT	GCGNAGACC	CACATGCTAC	AAAGCGGGCA	AAATNAGTG	ACAGATGACC

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of a 110 kd species when not reduced and a single 55 kd band in the presence of reducing agent. This indicates that on transfected COS cells, CD27 is a disulfide linked homodimer comprised of 55 kd monomers, similar to the forms precipitated from T lymphocytes. (Bigler et al., J. Immunol. 141:21-28 (1988); Stockinger et al., Leukocyte Typing II, Vol. I:513-529 (1986); Van Lier et al., Eur. J. Immunol. 18:811-816 (1987)).

CD27 is a T lymphocyte activation antigen. Its structure suggests that it may function as the receptor for a lymphokine or growth factor. The recognition of CD27 causes T cell proliferation and increased expression of certain genes needed for the helper and effector functions of the T cell. The expression of CD27 on T cells increases two to five fold with stimulation by phytohemagglutinin (PHA) or anti-CD3 monoclonal antibodies and the addition of at least one CD27 monoclonal antibody can augment PHA stimulated proliferation of T cells (Bigler and Chiorazzi, Leukocyte Typing II, Vol. I:503-512 (1986); Van Lier, (1987)). T cells positive for CD27 have been found to provide help to B cells for IgM synthesis and secrete Il-2 when appropriately stimulated (Van Lier et al., Eur. J. Immunol. 18:811-816 (1988)).

The ability to interfere with the binding of CD27 positive T cells with antigen presenting cells, or the ability to cause such binding to occur on surfaces other than lymphocyte cells, can be useful in diagnostics and therapy. For example, a fusion protein of CD27 and a receptor ligand fixed to a substrate will be useful in detecting the presence of a particular antigen in body fluids. A soluble CD27 fusion protein will be useful to prevent undesired T cell proliferation, for example, in certain autoimmune diseases.

Example XIV The Isolation and Molecular Cloning of the Two cDNA Clones Encoding T Lymphocyte-specific Leu8 Antigens

Two cDNA clones encoding Leu8 determinants were isolated from a human T cell library by the method of the present invention.

5 The nucleotide sequence of the cDNA was determined by dideoxynucleotide chain termination as described, supra. The DNA sequence analyses (Table 6) shows that the longer insert of the two contains 2,350 residues, whereas the shorter lacks 436 internal residues but is otherwise identical. The entire sequence of the longer clone is shown, with the portion deleted from the shorter clone overlined. The predicted amino acid
10 sequence is shown below the nucleotide sequence. Sites of potential N-linked glycosylation are designated --CHO-- and the proposed transmembrane region for the longer form is doubly underlined.

15 DNA blot hybridization of fragmented human T cell genome showed a pattern consistent with a single copy gene. RNA blot hybridization revealed a major transcript of 2.4 kb in peripheral blood mononuclear cells, tonsillar B cells, and several lymphocytic cell lines; and a minor transcript of 2.0 kb, present in peripheral blood mononuclear cells, and the Jurkat and HSB-2 leukaemic T cell lines.

20 The deduced protein encoded by the larger insert (the conventional form) bears a strongly hydrophobic putative membrane spanning domain near its C terminus, followed by several positively charged residues resembling a cytoplasmic anchor
25 sequence. The protein is closely related to the recently described murine Mel-14 homing receptor (Lasky et al., Cell 56:1045-1055 (1989); Siegelman et al., Science 243:1165-1172 (1989)).

30 The protein encoded by the shorter insert (the phospholipid anchored form) bears a weakly hydrophobic C-terminal domain characteristic of surface proteins that are attached to the cell

TABLE 6

1	CTCCCTTTGG	GCAAGGACCT	GAGACCCTTG	TGCTAAGTCA	AGAGGCTCAA	TGGGCTGCAG	AAGAACTAGA	GAAGGACCAA
					M	G	C	R
						R	T	R
						E	G	P
						S		
81	GCAAGGCCAT	GATATTTC	CA	TGGAATATC	AGAGCACC	CA	GAGGACTTA	TGGAACATCT
	K	A	M	I	F	P	W	K
							C	Q
							S	T
							Q	R
							D	L
							W	N
							I	F
							K	L
							W	G
							W	T
161	ATGCTCTGTT	GTGATTTCT	GGCAGATCAT	GGAACCGACT	GCTGGAATTA	CCATTATCT	GAAAGACCA	TGAACTGGCA
	M	L	C	C	D	F	L	A
							H	H
							G	T
							D	C
							W	T
							Y	H
							S	E
							K	P
							M	N
							W	Q
241	AAGGCTAGA	AGATTCTGCC	GAGACAAATTA	CACAGATTTA	GTTGCCATAC	AAAACAAAGC	GGAATTTAG	TATCTGGAGA
	R	A	R	R	F	C	R	D
							N	Y
							T	D
							L	V
							A	I
							Q	N
							K	A
							E	I
							E	Y
							L	E
							K	
321	(1)	AGACTCTGCC	TTTCAGTGGT	TCTTACTACT	GGATAGGAAT	CCGGAAGATA	GGAGGAATAT	GGACGTTGGT
		T	L	P	F	S	R	S
							Y	Y
							W	I
							G	I
							R	K
							I	G
							G	I
							W	T
							W	V
							G	T
							N	
401	AAATCTCTCA	CTGAGAGAGC	AGAGAACTGG	GGAGATGGTG	AGCCCAACAA	CAAGAGAAC	AAGGAGACT	GCGTGAGAT
	K	S	L	T	E	E	A	E
							N	W
							G	D
							G	E
							P	N
							N	K
							K	N
							K	E
							D	C
							V	E
							I	
481	CTATATCAAG	AGAAACAAG	ATGCAGGCAA	ATGGAAAGAT	GAGGCTGCC	ACAACTTAA	GGCAGCCCTC	TGTTACACAG
	Y	I	K	R	N	K	D	A
							G	K
							W	N
							D	A
							C	H
							K	L
							K	A
							A	L
							C	Y
							T	A
561	CTTCTTGCCA	GCCCTGGTCA	TGCAGTGGCC	ATGGAAGATG	TGTAGAAATC	ATCAATATTT	ACAACTGCAA	CTGTGATGTG
	S	C	Q	P	W	S	C	S
							G	H
							G	E
							C	V
							E	I
							I	N
							Y	T
							C	N
							C	D
							V	
641	GGGTACTATG	GGCCCCAGTG	TCAGTTTGTG	ATTCAAGTGTG	AGCCTTTTGA	GGCCCCAGAG	CTGGGTACCA	TGAACTGTAC
	G	Y	Y	G	P	Q	C	Q
							F	V
							I	Q
							C	E
							P	L
							E	A
							P	E
							L	G
							T	M
							D	C
							T	
721	TCACTCTTTG	GGAAACTTCA	GCTTCAGCTC	ACAGTGTGCC	TTCAAGCTGCT	CTGAAGGAAC	AAACTTAACCT	GGGATTGAAG
	H	S	L	G	N	F	S	Q
							C	A
							F	S
							C	S
							E	G
							T	N
							L	T
							G	I
							E	E
801	AAACCACTG	TGACCAATTT	GGAAGCTGTT	CATCTCCAGA	ACCAACCTGT	CAAGTGATTC	AGTGAGGCC	TCTATCAGA
	T	T	C	G	P	F	G	N
							W	S
							S	P
							E	P
							T	C
							Q	V
							I	Q
							C	E
							P	L
							S	A
881	CCAGATTGG	GGATCATGAA	CTGTAGCCAT	CCCCTGCCA	GCTTCAGCTT	TACCTCTGCA	TGTACCTTCA	TCTGCTGAGA
	P	D	L	G	I	M	N	C
							S	H
							P	L
							A	S
							F	S
							F	T
							S	A
							C	T
							F	I
							C	S
							E	

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--CHO--

961 AGGAAGTGAAG TTAATTGGGA AGAAGAAAAC CATTGTGTGA TCATCTGGAA TCTGGTCAAA TCCTAGTCCA ATATGTCAAA
G T E L I G K K K T I C E S S G I W S N P S P I C Q K

1041 AATTGGACAA AAGTTTCTCA ATGATTAAAG AGGCTGATTA TAAACCCCTC TTCATTCCAG TGGCAGTCAT GGTACTGCA
L D K S F S M I K E G D Y N P L F I P V A V M V T A

1121 TTCTCTGGGT TGGCATTAT CATTGGCTG GCAAGAGAT TAAAAAAGG CAAGAAATCC AAGAGAATA TGAATGACC
F S G L A F I I W L A R R L K K G K K S K R S M N D P

1201 ATATTAAATC GCCCTTGGTG AAGAAAATT CTGGAATAC TAAAAATCAT GAGATCCTTT AATCCTTCC ATGAAAAGTT
Y *

1281 TTGTGTGGTG GCACCTCCTA CGTCAAAACAT GAAGTGTGT TTCTTCAGTG CATCTGGGA GATTCTACC TGACCAACAG

1361 TTCCCTTCAGC TTCCATTTCG CCCCTCATTT ATCCCTCAAC CCCCAAGCCA CAGGTGTTA TACAGCTCAG CTTTGTCT

1441 TTCTTGAGGA GAAACAATA AGACCATTA GGAAGAGAT TCATCTGGA TATTAAGATG GCTGACTTTG CTCTTCTTG

(293)
F L

1521 ACTCTTGTTC TCAGTTTCAA TTCAGTCTG TACTGATGA CAGACACTTC TAAATGAAGT GCAAATTGGA TACATATGTC
T L V F S F N S V L Y L M T D T S K *

1601 AATATGGAAT CAGTTTCTT GCAGATCAAA TTTCACGTCG TCTTCTGTAT ACTGTGAGG TACACTCTTA TAGAAGTTTC

1681 AAAAAGTCTA CGCTCTCCTT TCTTCTTAC TCCAGTGAAG TAAAGGGGTC CTGCTCAAGT TGAAGAAGTC CTATTGACAC

1761 TGTAGCCTCG CCGTCTGTGA ATTGACCANT CCTATTAAAC TGGCTTCAGC CTCCCACTCT TCTTCAGCCA CCTCTCTTT

1841 TCAGTTGGCT GACTTCGACA CCTAGCATCT CATGAGTGGC AAGCAAAAAG AGAGAAGAGA GAATAAGCCT GCGCTGTTTT

1921 TTAGTTTGGG GGTTTGCTG TTTCCTTTTA TGAGACCANT TCCTATTCTT TATAGTCANT GTTCTTTTA TCACGATATTT
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2001 ATTAGTAAAG AAACATCACT GAATGCTAG CTGCAAGTGA CATCTCTTTG ATGTCAATAG GAAGAGTTAA AACAGGTGA
2081 GAAATTCCTT GATTACAAAT GAAATGCTCT CCTTCCCTT GCCCCAGAC CTTTATCCG ACTTACCTAG ATTCTACATA
2161 TTCTTTAAAT TTCATCTCAG GCCTCCCTCA ACCCCACAC TTCTTTTATA ACTAGTCTT TACTAATCA ACCCATGATG
2241 AGCTCCCTCT CCTGGCTTCT TACTGAAGG TTACCCTGTA ACATGCAATT TTGCAATTGA ATAAAGCCTG CTTTTAAGT
2321 GTTAAATAAA AAAAAAAAAA AAAAAAAAAA

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A74

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membrane by covalent linkage to a phosphatidylinositol-substituted glycan.

Monoclonal antibodies TQ1 (Reinherz et al. (1982) *J. Immun.* 128:463-468) and Mel-14 (Gallatin et al. (1983) *Nature* 304:30-34) have been observed to react with COS cells transfected with either Leu8 clone.

5 The presence or absence of Leu8 on CD4⁺ T lymphocytes identifies suppressor-inducer and helper-inducer CD4⁺ T cell subsets. Leu8 is a homing receptor, allowing T cells to adhere to the specialized post-capillary endothelium of peripheral lymph nodes. The presence or absence of Leu8 classifies the T cell in terms of homing potential and tissue distribution. Serological studies have indicated that Leu8 is a marker of resting lymphocytes in peripheral lymph nodes (Poletti et al., *Hum. Pathol.* 19:1001-1007 (1988)). Activation of T cells by phorbol ester plus PHA results in reduced Leu8 expression and transcripts, with the reduction in Leu8 expression being more rapid than the reduction of Leu8 transcripts. It therefore appears that surface Leu8 is lost more rapidly than predicted by RNA turnover, possibly by shedding of the phosphatidylinositol-linked form (Ferguson & Williams, *A. Rev. Biochem.* 57:285-320 (1988)). Among peripheral T cells, the CD4⁺ Leu8⁻ subset provides help for B cell IgM and IgG synthesis (Reinherz et al., *J. Immun.* 128:463-468 (1982); Gatenby et al., *J. Immun.* 129:1997-2000), whereas CD4⁺ Leu8⁺ cells have been found to directly inhibit pokeweed mitogen-induced IgG synthesis (Kanof et al., *J. Immun.* 139:49-54 (1987)). It therefore appears that CD4⁺ Leu8⁺ cells, activated to provide help for B cell Ig synthesis, exit the nodes and circulate peripherally to encounter antigen-presenting cells.

The ability to interfere with the binding of Leu8⁺ T cells to antigen presenting cells, or the ability to cause such binding to occur on surfaces other than lymphocyte cells, can be useful in diagnostics and therapy. For example, the level of activated

Leu8⁺ T cells relative to resting Leu8⁺ cells could serve as a measure of immune response to a particular antigen.

The extracellular domain of the Leu8 transmembrane protein, which mediates adhesion to specialized endothelial cells of lymph nodes, has been observed to be quite specific in its recognition of the lectin ligand, sulfated galactosyl ceramide (sulfatide). Modification of the specificity of this binding could serve to regulate the homing potential of resting T cells. Soluble forms of Leu8 can act as anti-inflammatory agents by reducing lymphocyte migration.

Example XV The Isolation and Molecular Cloning of cDNAs Encoding CD44 Antigens

CD44 is a polymorphic integral membrane protein. Immunochemical and RNA blot data have supported the existence of two forms of CD44: a mesenchymal form expressed by hematopoietic cells and an epithelial form weakly expressed by normal epithelium but highly expressed by carcinomas.

To isolate a cDNA clone encoding hematopoietic CD44 (Stamenkovic et al., Cell 56:1057-1062 (1989)), libraries prepared from the histiocytic lymphoma cell line U937, the B lymphoblastoid line JY, the Burkitt's lymphoma line Raji, and the myeloid leukemia line KG-1 were transfected separately into COS cells by the DEAE-Dextran method, described supra. The cells were pooled 48 hours after transfection, incubated with anti-CD44 monoclonal antibodies J173 (Pesandro et al., J. Immunol. 137:3689-3695 (1986)), and panned on dishes coated with goat antimouse affinity purified antibody. After several washes, the adherent cells were lysed, and episomal DNA was purified and transformed into E. coli. After two similar rounds of enrichment following spheroplast fusion, plasmid DNA recovered from three out of eight randomly picked colonies was found to direct the appearance of hematopoietic CD44 determinants on transfected COS cells.

Two of the three clones, CD44.5 and CD44.8, bore inserts of about 1.4 kb, while the third, CD44.4, contained an insert of about 1.7 kb. COS cells transfected with either of these clones reacted with anti-CD44 monoclonal antibodies J173, F-10-44-2 (Dalchau et al., Eur. J. Immunol. 10:745-749 (1980)) and the anti-Pgp-1 monoclonal antibody IM7 (Trowbridge et al., Immunogenetics 15:299-312 (1982)). Untransfected cells showed weak J173 reactivity.

The nucleotide sequence of the hematopoietic CD44.5 cDNA (Table 7) consists of 1354 residues terminating in a short poly(A) tail 19 base pairs downstream from a CATAAA sequence. The ATG encoding the first methionine is embedded in a consensus initiation sequence and followed by 19 predominantly hydrophobic residues resembling a secretory signal peptide sequence. Cleavage of this peptide would yield a mature protein of 341 residues with a predicted relative molecular mass of 37.2 kd. The extracellular amino terminal domain of 248 residues is followed by 21 predominantly hydrophobic amino acids corresponding to the predicted transmembrane domain (doubly underlined) and a 72 residue hydrophilic (cytoplasmic) domain. The discrepancy between the predicted mass of the protein backbone and the deglycosylated forms observed in immunoprecipitates suggest that extensive O-linked glycosylation is present. The extracellular domain has six potential N-linked glycosylation sites, indicated in Table 7 by a --CHO-- designation, and is rich in serine and threonine residues (22% in aggregate). The dipeptide SG that forms the minimal attachment site of serine-linked chondroitin sulfate in proteoglycan proteins appears at residues 160, 170, 211 and 238 in the predicted extracellular domain; these potential glycosylation sites are underlined.

RNA blot hybridization revealed three major messages of 1.6, 2.2 and 5.0 kb in a variety of hematopoietic cell lines, including the B lymphoblastoid line CESS, the T cell leukemias

TABLE 7

1 CCAGCCTCTG CCAAGTTGCG TCCGCCATCC TCGTCCCGTC CTCGCCCGGC CCCTGCCCGC CGCCCAAGGA TCCTCCAGCT
 81 CCTTTCGCCC GCGCCCTCCG TTGCTCCCG ACACCATGGA CAGTTTGG TGGACGCGAG CCTGGGAGCT CTGCCCTGTC
 M D K F W W H A A W G L C L V
 161 CCGCTGAGCC TGGCGAGAT CGATTGGAAT ATAACTGCC GCTTTCAGG TGTATTCAC GTGAGAAA ATGCTGCTA
 P L S L A Q I D L N I T C R F A G V F H V E K N G R Y
 --C HO--
 241 CAGCATCTCT CGGAGGAGG CCGCTGACCT CTGCAGGCT TTCATAGCA CCTTCCCGAC ATGCGGAG ATGAGAAAG
 S I S R T B A A D L C K A F N S T L P T M A Q M E K A
 321 CTCTGAGCAT CGGATTGAG ACCTGCAGGT ATGGTTCAAT AGAAGGCAAT GTGGTATTC CCCGATCCA CCCCACTCC
 L S I G F E T C R Y G F I E G H V V I P R I H P N S
 --CHO--
 401 ATCTGTGCAG CAACACACAG AGGGGTGTAC ATCTCAACAT ACACACCTTC CCAATGCTT TGTATGACC NATTACCATA ACTATTGTTA
 I C A A N N T G V Y I L T Y N T S Q Y D T Y C F N A S
 --CHO--
 481 AGCTCCACCT GAGAGATTT GTACATCAGT CACAGACCTG CCCAATGCTT TGTATGACC NATTACCATA ACTATTGTTA
 A P P E E D C T S V T D L P N A F D G P I T I T I V N
 --CHO--
 561 ACCGTGATGG CACCCGCTAT GTCCAGAAAG GAGATATCAG AACGATCTT GAGACATCT ACCCCAGCAA CCCTACTGAT
 R D G T R Y V Q K G E Y R T N P E D I Y P S N P T D
 641 GATGACGTGA GCAGCGGCTC CTCGAGTGA AGGAGCAGCA CTTCAGGAG TTACATCTTT TACACCTTTT CTACTGTACA
 D D V S S G S S S E R S S T S G G Y I F Y T F S T V H

 721 CCCCATCCCA GACGAGACA GTCCCTGGAT CACCGACAGC ACAGACAGAA TCCCTGCTAC CAGAGACCAA GACACATTC
 P I P D E D S P W I T D S T D R I P A T R D Q D T F H
 801 ACCCCAGTGG GGGTCCCAT ACCACTCATG AATCTGATC AGATGACAC TCACATGGGA GTCANAGAG TGGACGAAAC
 P S G G S H T T H E S E S D G H S H G S Q E G G A N
 881 ACACCTCTG GTCTATAG GACACCCCA ATTCAGAT GGCTGATCAT CTGGCATCC CTCTGGGCT TGGCTTGAT
 T T S G P I R T P Q I P E W L I I L A S L L A L A L I

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TABLE 7 (Page 2)

961 TCTTGCAGTT TGCATTGCAG TCACACAGTCG AAGAAGGTGT GGGCAGAGGA AAAAGCTAGT GATCAACAGT GGCATGGAG
 L A V C I A V N S R R R C G Q K K K L V I N S G N G A
 =====
 1041 CTGTGGAGGA CAGAAAGCCA AGTGGACTCA ACCGAGAGGC CAGCAGTCT CAGGAATGG TGCATTGTTGGT GAAACAGAGAG
 V E D R K P S G L N G E A S K S Q E M V H L V N K E
 1121 TCGTCAGAAA GTCCAGACCA GTTTATGACA GGTGATGAGA CAAAGAACCT GCAGAAATGTG GACATGAGGA TTGGGGTGT
 S S E T P D Q F M T A D E T R N L Q N V D M K I G V *
 1201 ACAACCTACAC CATTAATCTTG GAAGAAACA ACCGTTGTAA ACATTAACCAT TACAGGAGC TGGGACACTT AACAGATGCA
 1281 ATGTGCTACT GATTGTTCA TTSCGAATCT TTTTACCAT AAAATTCTCT ACTCTTTTG TTAAAAAAA AAAA 1354

--C HO--

1
 Gatt
 AB

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HUT-102 and HPB-ALL, lymphokine activated T cells, tonsillar B cells and the histiocytic lymphoma U937.

Immunoprecipitation of CD44 from transfected COS cells reveals that the mesenchymal or hematopoietic form of CD44 is about 80-90 kd. Hematopoietic CD44 transfected into a B cell line has been observed to result in the binding of the CD44-bearing lymphocytes to rat lymph node stromal cells in primary culture, indicating that hematopoietic CD44 may play a role in lymphocyte homing. It has been shown that hematopoietic CD44 is an extracellular matrix receptor with affinity for collagens type I and VI (Stamenkovic et al., Cell 56:1057-1062 (1989)). Hematopoietic CD44 may also have a lymphocyte activation role.

The ability to interfere with the binding of hematopoietic CD44 to lymph node cells, or the ability to cause such binding to occur on other surfaces, can be useful in diagnostics and therapy. For example, modification of this binding can serve to regulate the homing potential of lymphocytes. Soluble forms of CD44 can have immunomodulatory activity.

To isolate a cDNA clone encoding the epithelial form of CD44, a cDNA library prepared from the colon carcinoma line HT29 was transfected into COS cells by the DEAE-Dextran method described supra. The cells were pooled 48 hours after transfection, incubated with anti-CD44 monoclonal antibody F-10-44-2 (Dalchau et al., Eur. J. Immunol. 10:745-749 (1980)) and panned on dishes coated with goat-anti-mouse affinity purified antibody. After several washes, the adherent cells were lysed, and episomal DNA purified and transformed into E. coli. After two similar rounds of enrichment following spheroplast fusion, as described supra, plasmid DNA recovered from seven out of ten randomly picked colonies was found to direct the appearance of epithelial CD44 determinants on transfected COS cells. All seven of the positive clones bore cDNA inserts of about 2.4 kb.

Restriction enzyme analysis of the clone containing the epithelial cDNA insert showed that the coding sequence (Table 8) was enlarged relative to the hematopoietic CD44 insert by the addition of 496 base pair. DNA sequence analysis showed that the epithelial CD44 cDNA is quite similar to the CD44.5 cDNA, but encoded an additional extracellular domain of 165 amino acids, inserted about 140 residues upstream of the transmembrane section shared by both clones. The mature protein would comprise 493 residues.

RNA blot analysis has revealed that the epithelial CD44 transcripts comprise 2.2, 2.7 and 5.5 kb species. Epithelial CD44 isolated by immunoprecipitation has revealed that the glycoprotein is about 160 kd.

Transfected B cells expressing epithelial CD44 do not adhere to rat lymph node stromal cells in primary culture as do hematopoietic CD44 transfected lymphocytes. The epithelial CD44 is weakly expressed by normal epithelium but highly expressed by carcinomas. It is possible that an extracellular matrix receptor function of epithelial CD44 may promote tumor invasiveness.

The ability to interfere with the binding of epithelial CD44 with extracellular matrices can be useful in therapy or diagnostics. For example, interference of the epithelial CD44 binding to extracellular matrices can diminish the likelihood of metastasis in cancer patients. Soluble forms of CD44 can act to prevent metastatic cells from "homing" to lymph nodes.

Example XVI The Isolation and Molecular Cloning of cDNA Encoding CD53 Antigens

CD53, the antigen recognized by antibodies MEM-53 (Hadam, M.R. (1989) in Leucocyte Typing IV, Knapp, B. et al. (eds.) Oxford Univ. Press, p. 674), HD77, HI29 and HI36, and 63-5A3, is a glycoprotein widely distributed among, but strictly restricted to, nucleated cells of the hematopoietic lineages (Stevanova, I., et al., (1989) in Leucocyte Typing IV, Knapp, B. et al. (eds.) Oxford Univ. Press, p. 678). CD53 is expressed by monocytes and

TABLE 8

1	CCAGCCTCTG	CAAGTTCGG	TCCGCCATCC	TGCTCCCGTC	CTCCGCCGGC	CCCTGCCCGG	CGCCCAAGGA	TCCTCCAGCT
81	CCTTTCGGCC	GCGCCCTCCG	TTGCTCCGG	ACACCATGA	CAAGTTTGG	TGGCAGCAG	CCTGGGACT	CTGCCTCGTG
				M D	K F W	H A A	W G L	C L V
161	CCGCTGAGCC	TGGCGCAGAT	CGATTTGANT	ATAACCTGCC	GCTTTGCAGG	TGTAATCCAC	GTGGAGAAA	ATGGTCGCTA
	R L S L	A Q I	D L N	I T C R	F A G	V F H	V E K N	G R Y
								--C HO--
241	CAGCATCTCT	CGGACGAGG	CCGCTGACCT	CTGCAAGCCT	TTCAATAGCA	CCTTGCCCA	AATGCCCA	ATGAGAAAG
	S I S	R T E A	A D L	C K A	F N S T	L P T	M A Q	M E K A
321	CTCTGAGCAT	CGGATTGAG	ACCTGCAGGT	ATGGGTTCA	AGAGGGCAT	GTEGTGATT	CCCGATCCA	CCCCACTCC
	L S I	G F E	T C R Y	G F I	E G H	V V I	P R I H	P N S
401	ATCTGTGCAG	CAACACAC	AGGGGTGTAC	ATCCTCAGAT	ACAACACCTC	CCAGTATGAC	ACATATTGCT	TCAATGCTTC
	I C A A	N N T	G V Y	I L T Y	N T S	Q Y D	T Y C F	N A S
481	AGCTCCACCT	GAAGAGATT	GTACATCACT	CACAGACCTG	CCCAATGCCCT	TTGATGACC	AATTACATA	ACTATTGTTA
	A P P	E E D C	T S V	T D L	P N A F	D G P	I T I	T I V N
561	ACCGTGATGG	CACCCGCTAT	GTCCAGAAAG	GAGAAATACAG	AACGAATCCT	GAAGACATCT	ACCACAGCA	CCCTACTGAT
	--CHO--							
	R D G	T R Y	V Q K G	E Y R	T N P	E D I Y	P S N	P T D
641	GATGACGTGA	GCAAGCGCTC	CTCCAGTGA	AGAGCAGCA	CTTCAGGAGG	TTACATCTTT	TACACCTTTT	CTACTGTACA
	D D V S	S G S	S S E	R S S T	S G G	Y I F	Y T F S	T V H
721	CCCCATCCCA	GACGAGACA	GTCCCTGGAT	CACCGACAGC	ACAGACAGAA	TCCCTCGTAC	CAATATGGAC	TCCAGTCATA
	P I P	D E D S	P W I	T D S	T D R I	P R T	N M D	S S H S
801	GTACACGCT	TCAGCCTACT	GCAATCCAA	ACACAGGTTT	GGTGAGAT	TTGGACAGGA	CAGGACCTCT	TTCAATGACA
	T T L	Q P T	A N P N	T G L	V E D	L D R	T G P L	S M T
881	ACGCAGCAGA	GTATTTCTCA	GAGCTTCTCT	ACATCACATG	AAGGCTTGA	AGAGATTA	GACCATCCA	CAACTTCTAC
	T Q Q S	N S Q	S F S	T S H E	G L E	E D K	D H P T	T S T
961	TCTGACATCA	AGCAATAGGA	ATGATGTAC	AGGTGAGAG	AGAGACCCA	ATCATTTCTGA	AGGCTCACT	CATTACTG
	L T S	S N R N	D V T	G G R	R D P N	H S E	G S T	H L L E

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TABLE 8

1041 AAGTTATAC CTCTCATTC CCACACACGA AGGAAGCAG GACCTTCATC CCAGTGACCT CAGCTAAGAC TGGGTCTTT
 G Y T S H Y P H T K E S R T F I P V T S A K T G S F
 1121 GGAGTACTG CAGTTACTGT TGGAGATTCC AACTCTAATG TCAATCGTTC CTATACAGGA GACCAAGACA CATTCACCC
 G V T A V T V G D S N S N V N R S L S G D Q D T F H P
 1201 CAGTGGGGG TCCCATACCA CTCATGATC TGAATCAGAT GGACACTCAC ATGGAGTCA AGAAGGTGA GCNAACACAA
 S G G S H T T H G S E S D G H S H G S Q E G G A N T T
 1281 CCTCTGTCC TATAAGACA CCCCAATTC CAGAATGGCT GATCATCTTG GCATCCCTCT TGGCCTTGGC TTGATTTCT
 S G P I R T P Q I P E W L I I L A S L L A L A L I L

 --CHO--
 1361 GCAGTTTGA TTGCACTCAA CAGTCGAAGA AGGTGTGGC AGAAGAAAA GCTAGTGATC AACAGTGGA ATGAGCTGT
 A V C I A V N S R R R C G Q K K K L V I N S G N G A V
 =====
 1441 GGAGACAGA AAGCCAGTG GACTCAACGG AGAGCCAGC AAGTCTCAGG AAATGGTGCA TTGTGTAAC AAGAGTCTG
 E D R K P S G L N G E A S K S Q E M V H L V N K E S S
 1521 CAGAACTCC AGACCAGTTT ATGACAGCTG ATGAGACAAG GAACTGCAG AATGTGACA TGAAGATTGG GGTGTACAC
 E T P D Q F M T A D E T R N L Q N V D M K I G V *
 1601 CTACACCATT ATCTTGAAA GAACAAAGT TGAACAATA ACCATTACAG GGGAGCTGG ACACTTAACA GATGCATGT
 1681 GCTACTGATT GTTTCATTTC GAATCTATTA TAGCATAAAA TTTTCTACTC TTTTGTITT TTGTGTTTG TTCTTTAAG
 1761 TCAGGTCCA TTTGTAATA CAGCATTGCT TTCTGAATT AGGCCCAAT TAATAATCAG CAAGAAITTT GATCGTTCA
 1841 GTTCCCCACT TGGAGGCTT TCATCCCTCG GGTGTCTAT GATGGCTTC TAACAAAAAC CTACACATA GTTATTCCTG
 1921 ATGCGCAACC TTGCCCCCA CCAGCTAAG ACATTTCAG GGTAAATAG GCCTGTCTT GGGAGGAAT TTGAATGGT
 2001 CATTTGCCC TTCCATTAGC CTAACTCCCTG GCATTTGCTT TCCACTGAGG TTGGGGTTG GGGTACTA GTTACACATC
 2081 TTCACAAGAC CCCCTCTAGA AATTTTTCAG ATGCTTCTGG GAGACACCA AAGGTAACT CTATTATCT GTAGTAACT
 2161 ATTTATCTGT GTTTTGAAA TATTAAACC TGAATCAGTC CTTTATTC A GTATTAATTT TTAAGTTTAC TTGTACAGAG
 2241 GCACAAAAG GTTTAAACT GATTCATTAAT AAATATCTGT ACCTTCTCG AAAAAAAA AAAAAAAA

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macrophages, by granulocytes, dendritic cells, osteoclasts and osteoblasts, and by T and B cells from every stage of differentiation.

To obtain a cDNA clone encoding CD53, cDNA libraries constructed from peripheral blood lymphocytes and the promyelocytic tumor cell line HL60 were transfected into COS cells by the DEAE-Dextran method, described supra. The cells were pooled 48 hours after transfection, incubated with monoclonal antibodies MEM-53, and panned as described in Seed and Aruffo, Proc. Natl. Acad. Sci. USA 84:3365-3369 (1987) and Aruffo and Seed, Proc. Natl. Acad. Sci. USA 84:8573-8577 (1987). After two subsequent rounds of enrichment following spheroplast fusion, plasmid DNA recovered from single colony isolates was transfected into COS cells, and scored for CD53 expression by immunofluorescence.

Two of eight transfectants were positive, each bearing an insert of about 1.5 kb. COS cells transfected with either clone reacted with each of the antibodies MEM-53, HI29, HI36 and 63-5A3.

To isolate CD53 from peripheral blood lymphocytes and transfected COS cells for purposes of comparison, the lymphocytes and transfected COS cells were surface labeled with ^{125}I using lactoperoxidase and H_2O_2 , and then lysed in a lysis buffer of 50 mM Tris-HCl pH 8.0 containing 1% NP40, 150 mM NaCl, 5 mM MgCl_2 , 5 mM KCl, 20 mM iodoacetamide and 1 mM phenylmethylsulfonyl-fluoride. Cells were solubilized at a concentration of 2.5×10^7 cells/ml in lysis buffer for 45 minutes then centrifuged at 12,000 g. After preclearing with goat anti-mouse immunoglobulin beads (Cappel, Malvern, PA), immunoprecipitations were performed with monoclonal antibodies MEM53 or 63-5A3 and protein A-Sepharose CL-4B (Sigma, St. Louis, MO) as described by Schneider et al. (J. Biol. Chem. 257:10766 (1982)). Immunoprecipitates were eluted in SDS- sample buffer and analyzed on 12.5% acrylamide gels containing sodium dodecyl sulfate.

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5 A broad band of radioiodinated protein ranging in mass from 34 kd to 42 kd was obtained from peripheral blood lymphocytes either with MEM-53 or 63-5A3 monoclonal antibodies, comparable to the band obtained from CD53-transfected COS cells, which extended from 36 kd to 46 kd. The higher molecular mass in COS cells is atypical, as cell surface proteins recovered from transfected COS cells usually display unchanged or lower molecular mass than those found on the cell from which the cDNA clone originated (Aruffo and Seed, Proc. Natl. Acad. Sci. USA 10 84:3365-3369 (1987)). Approximately 15 kd of mass are liberated from the glycoprotein by digestion with endoglycosidase F (N-glycanase), whereas treatment with neuraminidase and O-glycanase has no effect on the apparent molecular mass (Hadam, M.R. (1989) in Leucocyte Typing IV, Knapp, B. et al. (eds.) Oxford Univ. Press, p. 674; Stevanova et al., in Leucocyte Typing IV, Knapp, B. et al. (eds.) Oxford Univ. Press, p. 678). An additional faint band of 20 kd, possibly unglycosylated precursor, was detected in immunoprecipitates of transfected COS cells, but was absent from immunoprecipitates of peripheral blood lymphocytes.

20
25 Blot hybridization of genomic DNA from the T cell line PEER digested with several enzymes revealed a pattern consistent with a single copy gene. RNA blot analysis revealed a single 1.8 kb mRNA derived from B, T, and myeloid cell lines and from peripheral blood lymphocytes. The level of expression was comparable in the different cell lines except in THP1 cell line, which had little CD53 mRNA. CD53 transcripts are more abundant in peripheral blood lymphocytes than in cultivated cell lines, consistent with the higher surface expression of CD53 among these cells.

30 The nucleotide sequence of CD53 cDNA was determined by dideoxynucleotide chain termination as described, supra, using synthetic oligonucleotide primers. The sequence of the CD53 insert consists of 1452 nucleotides (Table 9), and terminates close to two overlapping AATAAA motifs (singly underlined). The 35 3' noncoding sequence contains three examples of the ATTTA

sequence (indicated by quotation marks), which has been shown to mediate mRNA instability (Shaw and Kamen, Cell 46:659 (1986)). An open reading frame beginning at residue 74 encodes a protein of 219 amino acids with a predicted molecular weight of 24,340 kd. The predicted polypeptide is unusual in that it bears four major hydrophobic segments (doubly underlined), three of which fall in close proximity near the amino terminus of the molecule. The first hydrophobic segment is atypically long for either a signal sequence or a simple transmembrane alpha helix, and contains three cysteine residues and a glycine located in the middle. Both cysteine and glycine have been found to immediately precede the signal cleavage site (von Heijne, Nucleic Acids Res. 14:4683 (1986)), suggesting that the amino terminus of the mature protein begins in the middle of the first hydrophobic domain. Some support for this view is afforded by the finding that the size of the polypeptide backbone of ME491, a related type III integral membrane protein, discussed infra, is smaller than the size predicted from the cDNA sequence, possibly as a consequence of signal peptide excision. Because there are only two potential sites for N-linked glycan addition (indicated in Table 9 by --CHO-- designations), located between the third and fourth hydrophobic segments, the carboxyl terminus must lie inside the cell, as well as the short hydrophilic portion between the second and third hydrophobic segments. If the amino terminus is not processed, it must likewise remain intracellular.

CD53 is a Type III integral membrane protein related to three other membrane proteins: ME491 antigen, a melanoma protein whose increased expression correlates well with tumor progression (Hotta et al., Cancer Res. 48:2955 (1988)); CD37, an extensively glycosylated antigen predominantly expressed on B cells, but not B cell lineage specific (Schwartz et al., J. Immun. 140:905 (1988); and S5.7, an unglycosylated antigen broadly expressed on cells of hematopoietic lineage (Pressano et al., Cancer Res. 43:4812 (1983)). In addition, CD53 is distantly related to E. coli lac Y permease, a type III integral membrane protein which ferries lactose into the bacterial cell. CD53 transcripts in

peripheral blood lymphocytes increase in prevalence following mitogenic stimulation by PHA, suggesting that the protein may be involved in the transport of factors essential for cell proliferation.

5 Among the molecules with broad reactivity in the hemopoietic system, CD53 presently holds the widest reactivity as well as the strictest restriction to hematopoietic cells. Anti-CD53 antibodies are a useful tool for the identification of hematopoietic neoplasms, and may prove helpful for identifying morphologically poorly defined cells, for example in spleen or
10 bone marrow primary cultures.

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed within the scope of this invention.

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TABLE 9

1	CTCAAGATA ATCACTAAT TCTGCCGAA GGA CTGAGGA ACGTGCCCTG GAAAAGGCA AGAATATCAC GGCATGGCA M G M
81	TGAGTAGCTT GAACTGCTG AAGTATGTC TGTTCCTT CAACCTGCTC TTTTGATCT GTGGCTGCTG CATTTGGGC S S L K L L K Y V L F F F N L L F W I C G C I L G =====
161	TTTGGATCT ACCCTGCTGAT CCACACAAAC TTGGAGTGC TCTTCATTA CCTCCCTCC CTACGCTGG GCAATGTCTT F S I Y L L I H N N F G V L F H N L P S L T L G N V F =====
241	TGTCATCGTG GGCTCTATTA TCATGTAAGT TGCCTTCCTG GGCTGCATGG GCTCTATCAA GGAACAAG TGTCTGCTA V I V G S I I M V V A F L G C M G S I K E N K C L L M =====
321	TGTCGTTCTT CATCCTGCTG CTGATTAACC TCCCTGCTGA GGTGACCTTG GCCATCCTGC TCTTTGTATA TGAACAGAG S F F I L L L I I L L A E V T L A I L L F V Y E Q K =====
401	CTGAATGAGT ATGCGCTAA GGCTGAGCC GACAGCATCC ACCGTTACCA CTCAGACAT AGCACCAAG CAGCGTGGGA L N E Y V A K G L T D S I H R Y H S D N S T K A A W D --C HO--
481	CTCCATCCAG TCATTTCTGC AGTGTGTGG TATATATGCG ACCAGTGATT GGACCAAGTG CCCACACAGA TCTTGCCCT S I Q S F L Q C C G I N G T S D W T S G P P A S C P S --CHO--
561	CAGATCGAAA AGTGAAGGT TGCTATGCGA AAGCAAGACT GTGCTTCAT TCCAATTTCC TGTATATCGG AATCATCACC D R K V E G C Y A K A R L W F H S N F L Y I G I I T =====
641	ATCTGTGTAT GTGTGATTA GGTGTGGGG ATGTCCCTTG CACTGACCCCT GAACTGCCAG ATTGACAAA CCAAGCAGAC I C V C V I E V L G M S F A L T L N C Q I D K T S Q T =====
721	CATAGGGCTA TGATCTGAG TAGTCTCTG GTGAAGAGAC TTGTTTCATC TCCGGAATG CAATAACATT TATAGCATGA I G L * =====
801	AGCCCTACAT GATCACTGCA GGATGATCCT CCTCCCATCC TTTCCCTTTT TAGGTCCCTG TCTTATACAA CCAAGAGAT =====
881	GGGTGTGGC CAGGCACATC CCATCTCAG CAGCAAGACA ATCTTTCACT CACTGACGGC AGCAGCCTAG TCTCTCAAAG =====

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TABLE 9 (continued)

961 TGGTGAACT AATATCTGAG CATCTTTTAC ACAAGAGAGG CAAAGACAAA CTGGATTTA TGGCCCAACA TCAGAAGGTG
 1041 AACCCAGGAT ATGAATTTT GCATCTTCCC ATTGTCGAAT TAGTCTCCAG CCTCTAATA ATGCCAGTC TTCTCCCAA
 1121 AGTCNAGCNA GAGACTAGTT GAAGGAGTT CTGGGCCAG GCTCAGTGA CCATTGTAC AACCTCTGT TTCTCTTGA
 1201 CTAACTGCC TGGCTACAGG AATTACACAG TTCTTTCTT CCAAGGGCA AGATCTCAT TCAATTCTT TATTAGAGG
 1281 CCTTATTGAT GGTCTTAAAG TCTTCCAGA AAAAATAAT CCAGTATTT ATATCTGAT TTCAACCAGT CACTTAGCTG
 1361 ATNATCACAG TAAGNAGACT TCTGGTATTA TCTCTATC AGATAAGATT TTGTTAATGT ACTATTTTAC TCTCAATTA
 1441 ATAAAAACAGT TT 1452

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